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13. ABSTRACT *(Maximum 200 words)*

SUMMARY YEAR 1 - The first year of this contract was devoted to the conjugation of selected antivirals (ribavirin and PMEA) and immunomodulators (MDP) to neoglycoprotein carriers (poly-L-lysine or BSA). Our *in vivo* virus challenge studies suggest that MDP-BSA and Poly-L-lysine conjugates were more effective than free MDP in enhancing resistance to HSV-1 hepatitis and pneumonitis. Moreover, conjugated MDP was more effective in enhancing RES function than was free drug. In addition, conjugated PMEA was more effective than free drug in reducing virus titers in lungs of infected mice.

Squirrel monkeys were shown to be useful in the evaluation of an orally administered immunomodulating agent, CL 246738, as evidenced by the induction of serum interferon. Thus, this primate model was selected for the preclinical evaluation of promising new drugs (see below).

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A human macrophage antiviral assay was shown to be a valuable tool for *in vitro* evaluation of targeted antivirals and immunostimulants. This assay was used to study both RNA and DNA viruses and has allowed us to examine the biological activity of free and conjugated drugs. For example, we have shown that conjugation of the nucleoside analogue, PMEA, to poly-lysine could be accomplished without any loss of biological activity as determined by the *in vitro* HSV-1/macrophage assay.

SUMMARY YEAR 2 - Administration of multiple doses of Poly I:C-LC, Riker-3M or CL 246878 every other day for ten days did not prolong and in some cases even suppressed activation of immune functions. Nevertheless, administration of multiple doses of these drugs had no adverse effects on resistance to Banzi virus infection. Encapsulation of Poly I:C in liposomes resulted in a formulation which was more effective than free drug in augmenting immune functions and resistance to virus challenge. Poly I:C conjugated to dextran was superior to free Poly I:C-LC in inducing interferon and stimulating the reticuloendothelial system. In addition, Poly I:C-dextran conjugates did not have the apparent toxic effects on the reticuloendothelial system that were observed with comparable doses of Poly I:C-LC.

SUMMARY YEAR 3 - In this year we focused our attention on a comparison of the efficacy of various formulations of immunomodulators, the development of a dengue type 2 infection in squirrel monkeys and the mechanisms of Poly I:C induced resistance to virus infection *in vitro* and *in vivo*. In general, all formulations of Poly I:C (liposome-encapsulated; [Poly I:C-Lip], conjugation to dextran [Poly I:C-Dex]; or complexing with poly-L-lysine and carboxymethylcellulose; [Poly I:C-LC]) were more effective than free drug in induction and maintenance of serum interferon in mice and squirrel monkeys. Moreover, serum interferon appeared more rapidly in animals receiving conjugated

13. Abstract (Con't)

Poly I:C. In addition, Poly I:C-Dex conjugates appeared to less toxic than Poly I:C-LC as measured by reticuloendothelial system function.

Type 2 dengue virus (strain 16681) infects squirrel monkeys as evidenced by fever, viremia and induction of anti-dengue antibodies. This virus model provides an opportunity to evaluate the therapeutic potential of immunomodulating and antiviral agents in non human primates infected with an arbovirus and should contribute the preclinical data needed for the use of these drugs in man.

The *in vitro* antiviral activity of Poly I:C in macrophages is mediated solely by the production of interferon-β (IFN-β) which acts in an autocrine manner to induce resistance to infection and prevent development of cytopathic effects. The *in vivo* antiviral activity of Poly I:C against Banzi virus is also mediated through the production of IFN. Although macrophages, natural killer cells and T cells, which can be activated by IFN and Poly I:C, may play a secondary role, these cells do not appear to be essential determinants in the protective effects of Poly I:C against Banzi virus. IFN-α appears to be the predominant IFN type involved in resistance to Banzi virus *in vivo*. These results are in contrast to the role which IFN-β plays in the induction of macrophage intrinsic antiviral resistance following Poly I:C administration *in vitro*.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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__X__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__X__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

J. David Gazzo Eugene P. Mayer

PI - Signature Date 8/23/91

TABLE OF CONTENTS

LIST OF APPENDICES

Tables

Figures

Figure 3. Chemical structure of gluconoylated and mannosylated Poly-L-Lysine substituted with MDP or ribavirin residues.

Figure 4. Treatment of HSV-1 induced hepatitis with MDP conjugates.

Figure 5. Treatment of HSV-1 induced pneumonitis with MDP conjugates.

Figure 6. RES stimulation by mannosylated Poly-L-lysine MDP conjugate.

Figure 7. Free versus conjugated PMEA in the treatment of human macrophages infected with HSV-1.

Figure 8. Phagocytosis by peritoneal exudate cells following oral administration of Riker-3M on day -2.

Figure 9. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M on days -2 and -4.

Figure 10. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M on days -2, -4 and -6.

Figure 11. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M on days -2, -4, -6 and -8.

Figure 12. Prophylactic activity of Riker-3M in Banzi encephalitis.

Figure 13. Prophylactic activity of CL 246738 in Banzi encephalitis.

Figure 14. Resistance to Banzi virus following five or seven i.v. injections with Poly I:C-LC.

Figure 15. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated Poly I:C on day -2.

Figure 16. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of free Poly I:C on day-2.

Figure 17. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated Poly I:C on day -2.

Publications resulting from this contract.

PROBLEM UNDER INVESTIGATION

A. BACKGROUND AND HYPOTHESIS

A number of clinically proven antivirals, such as amantadine, iododeoxyuridine, adenine arabinoside, acycloguanosine, ribavirin, and azidodeoxythymidine are currently in use. In addition, several potentially useful antivirals (*e.g.* S-HMPA, selenazole, WIN 5177, arildone, phosphonoformic acid) are currently under investigation. There are, however, toxicity problems associated with the use of antivirals at doses required to provide sustained drug levels in virus infected organs. Because of the many similarities in synthetic "machinery" and pathways used by both viruses and mammalian cells, these toxicity problems may be difficult to overcome. Furthermore, in some virus infections even the presence of a highly active antiviral in the infected target organ may not be sufficient to prevent or limit disease. Thus, a delivery system which can provide slow release of an antiviral, alone or in combination with other effector substances (*e.g.*, immunostimulants, antibody, interferon), at the site of primary virus replication may be highly desirable. We believe, that with the recent technological advances in the development of carrier vehicles designed to protect nontarget environments from the drugs they carry, and facilitate slow release of one or more drugs at sites at which they are needed, many of the problems currently associated with antiviral chemotherapy can be minimized.

A.1 Rationale and theoretical considerations for the use of targeted drug carriers

Successful drug use in medicine is often jeopardized by the failure of drugs that are otherwise active *in vitro* to act as efficiently *in vivo*. This is because in the living animal drugs must, as a rule, bypass or traverse organs, membranes, cells and molecules that stand between the site of administration and the site of action. In addition, drugs may be prematurely excreted or inactivated; thus, an effective therapeutic drug concentration may not be obtained at the site of infection. While this problem can be overcome by increasing dosage, this often results in toxicity. For example, a number of antiviral agents (ribavirin, adenine arabinoside, phosphonoformic acid, azidodeoxythymidine) have been developed which are highly effective *in vitro* in preventing virus replication and/or cell death; however, their systemic use in man is limited by the induction of toxic effects which occur at dosages required to maintain effective drug concentrations in the infected organ. In particular, sustained treatment often results in leukopenia and subsequent immunosuppression (1,2) which may affect the outcome of treatment since recovery from most viral infections

involves the cooperation of host immune responses. There is now growing optimism that these problems may be resolved or minimized with the use of carrier vehicles such as liposomes, monoclonal antibodies or conjugated proteins. These vehicles not only protect the immune system from the drugs they carry but also deliver and facilitate drug release at site(s) at which they are needed.

A prereq site for the successful use of antiviral carriers is that they rec nize the target site and release drug in an active form. Thus, th antiviral must be linked to a carrier in a stable fashion so that it remains attached throughout its journey from the site of inoculation to the target. For this to occur, the drug-carrier linkage must be resistant to the various enzymatic and physicochemical conditions prevailing in the bloodstream and extracellular fluids. This stability also implies that the drug remains inert as long as it is associated with its carrier so that it will be inactive prior to reaching the target area. A corollary of this requirement is that there be a mechanism by which the drug will be activated after reaching its destination. Three possibilities exist for the activation of carrier bound drugs: 1) endocytosis and release in acidic endosomes; 2) extracellular activation at cell surfaces by plasma membrane associated enzymes; and 3) intracytoplasmic penetration via transmembrane passage.

During the past decade, the use of carriers for the selective targeting of antitumor drugs has been advocated with increasing frequency. This has led to numerous reports on the association of drugs or toxins such as anthracyclines, methotrexate, bleomycin, chlorambicin, cytosine arabinoside (ara-C), and ricin with carriers such as DNA (3,4), liposomes (5,6), monoclonal antibodies (7-9), hormones (10,11), red blood cell ghosts (12), neoglycoproteins (13) and other proteins (14,15). Most of these carriers have the ability to selectively interact with target cell surfaces and are subsequently endocytosed and transferred to the lysosomal compartment where free drug is released when the bond between drug and carrier is hydrolyzed by lysosomal enzymes (16). In contrast, some liposomes deliver their contents directly into the cytoplasm following fusion with the target cell membrane (17). This mode of delivery is useful for antivirals which are susceptible to lysosomal enzymes since membrane fusion is a mechanism through which drug contact with lysosomes can be bypassed. In addition, liposomes are useful in the delivery of poorly soluble agents such as arsenicals in the treatment of leishmaniasis (18) and lipophilic drugs such as MTP-PE in the treatment of herpesvirus infections (19,20). Moreover, they provide a means to deliver drugs that cannot be transported across cell membranes.

A.1.1 Liposomes

Four basic liposome designs: 1) multilamellar; 2) small unilamellar; 3) large unilamellar; and 4) vesicles produced by reverse phase evaporation, have been used as carrier systems for enhancing the pharmacological activity of drugs and the incorporation of macromolecules into cells. The most commonly used methods for the preparation of liposomes are the multilamellar vesicles originally described by Bangham *et al.* (21), and the small unilamellar vesicles initially described by Papahadjopoulos and Miller (22). The main drawbacks of these "classical" liposome preparations are the wide heterogeneity in size distribution and number of lamellae, the relatively low trapping efficiency in the aqueous space and/or their inability to encapsulate large macromolecules due to their small internal volume (23). In contrast, newer methods in the preparation of large unilamellar and reverse phase evaporation liposomes have resulted in carriers with larger internal volumes and better encapsulation properties. For example, a reverse evaporation process has been used to produce unilamellar vesicles capable of encapsulating substantial amounts of ara-C (24). These liposomes were composed of phosphatidylserine and cholesterol and were stable in serum. Furthermore, their distribution to lung, liver and spleen following intravenous inoculation could be controlled, in part, by altering one or more physical features (*e.g.*, size, membrane fluidity and charge,) or by embedding of glycoconjugates, proteins etc.

Comparison of pulmonary retention of liposomes of differing size, surface charge, and composition following iv injection revealed that large multilamellar or reverse phase evaporated liposomes arrested in the lung more efficiently than small unilamellar liposomes of identical lipid composition. In addition, multilamellar and reverse phase evaporation vesicles containing negatively charged amphophiles arrested in the lung more efficiently than neutral or positively charged structures which accumulated in the liver and spleen (25). Reverse phase evaporation carriers were superior to multilamellar carriers with respect to their loading capacity, however, the latter provided a more prolonged drug release.

Liposomes containing glycolipid have a preferential affinity for hepatocytes due to a galactose binding membrane lectin (26). Using liposomes which varied in size and galactosyl lipid content (galactocerebroside), Rahman *et al.* were able to show more selective targeting of small unilamellar vesicles to hepatocytes (27). Similarly, large liposomes containing galactocerebroside produced a substantial enhancement of liposome-encapsulated primaquine activity against the schizont stage of malarial parasite which is known to reside in hepatocytes (18).

In many instances, liposomes are avidly taken up by tissue associated macrophages which are present in most organs. Selective uptake of these vehicles by macrophages can be quite useful and can be exploited in some infectious diseases; thus, Alving *et al.* (18) used liposome-encapsulated antimonial drugs to treat leishmaniasis, a parasitic infection in which the macrophage is the primary target of infection. Encapsulated drug preferentially accumulated in these cells and killed the parasite with minimal toxicity for nontarget cells.

In addition to the examples cited above, liposomes have been used as carriers to enhance the immunogenicity of a variety of viral proteins including hepatitis B surface antigen, vesicular stomatitis virus glycoproteins, and adenovirus capsid proteins. Encapsulation of these proteins and inoculation into animals resulted in an increased production of serum neutralizing antibodies (28-30). In at least one study, in which purified major surface glycoproteins of VSV were encapsulated into liposomes, both specific humoral and cell mediated immunity were enhanced (28).

Recently, attempts to load antivirals into freeze-dried liposomes has resulted in improved encapsulation with minimal drug-leakage upon storage (personal communication, Dr. Peter van Hoogevest). This is particularly significant since it should now be possible to more easily load a variety of antivirals and/or immunostimulants into this type of carrier. We have recently encapsulated both ribavirin and muramyl tripeptide phosphatidylethanolamine (MTP-PE) into freeze-dried liposomes and have demonstrated their usefulness in the treatment of viral diseases. Thus, in the future, liposomes may become a more widely used vehicle for delivery of drugs in antiviral chemotherapy.

A.1.1.1 Liposome targeting of antivirals

One of the early applications of liposomes as a delivery vehicle for antivirals was made in the treatment of herpes keratitis with iododeoxyuridine (31). In this study, liposome encapsulated iododeoxyuridine was shown to be more effective than free drug in the treatment of both acute and chronic keratitis. In another study Kende *et al.*(32) showed a five-fold increase in liver concentrations of ribavirin following liposome encapsulation. In addition, encapsulated drug was more effective than free drug in the treatment of mice infected with the hepatotropic Rift Valley fever virus. More recently, we have demonstrated a five-fold increase in pulmonary concentrations of ribavirin when drug was encapsulated in liposomes and administered intravenously (20). Liposome-encapsulated ribavirin was more effective than free drug in protecting mice against lethal challenge with influenza virus. Thus, tissue targeting of antivirals with liposomes substantially increases their therapeutic index.

A.1.1.2 Liposome targeting of immunostimulants

Liposome encapsulated immunomodulators such as macrophage activating factor (MAF) and muramyl dipeptide (MDP), or its lipophilic analogue (MTP-PE), have been used to stimulate pulmonary macrophage activity and enhance host resistance to lung metastasis (26,33,34) and herpesvirus infection (19,20). Koff *et al.* (19) have shown that liposome-encapsulated MTP-PE was more effective than free MTP-PE in preventing death in a murine model of HSV-2 hepatitis. Similarly, we have demonstrated that liposome encapsulated MTP-PE was more effective that free drug in a murine model of HSV-1 pneumonitis (20). Protection observed with liposome encapsulated drug appeared, in part, to be due to enhanced drug localization in the liver and lungs of infected mice. In another study, it was shown that liposome-encapsulated MTP-PE was more effective than free drug in the healing of genital lesions induced by HSV-2 infection of guinea pigs. Therefore, liposome encapsulation of immunostimulants can be used to enhanced localization of drugs in various tissues, thus providing an effective stimulus for the activation of local macrophages.

A.1.2 Drug/Glycoprotein conjugates

Drug conjugated glycoproteins or neoglycoproteins (proteins such as serum albumin to which sugars have been attached) are promising targeting vehicles because they are easily prepared, biodegradable, and nontoxic. Fiume *et al.* (35-37) have shown that drugs can be stably conjugated to asialofetuin or galactosyl-terminating serum albumin and bind to lectin receptors on hepatocytes (38,39). Selective uptake of these intravenously administered drug conjugates resulted from the binding of carrier to galactosyl binding sites on hepatocyte membranes (35). Quite remarkably, drugs conjugated to serum albumin were selectively cleared by neighboring liver sinusoidal endothelial and Kupffer cells (37). Once inside these cells, the drug-conjugates entered lysosomes where hydrolytic cleavage released the active drug into the cytoplasm. In a similar study Monsigny *et al.* (40) were able to demonstrate the targeting of methotrexate by conjugation to fucosylated neoglycoproteins. These conjugates resulted in a more efficient killing of tumor cells by methotrexate.

As previously indicated, before a carrier can be effective, the link between drug and carrier must remain stable in the bloodstream and withstand the action of serum hydrolases. On the other hand, unless the drug is able to act in conjugated form at the cell surface, it must be released from the carrier after interaction of the conjugate with the target cell. In addition, its mode of

release must allow the drug to reach its subcellular target and interact effectively with it. Because the most general fate of molecules bound by surface receptors is to be internalized by endocytosis, and conveyed to lysosomes for digestion, an obvious way of insuring appropriate release of drug is to rely on lysosomal hydrolysis. This approach is of course limited to drugs that are not inactivated in the lysosomes and that can reach their biochemical target from the lysosomal compartment. Monsigny *et al.* have demonstrated that the stability of daunorubicin-carrier conjugates in serum can be enhanced by incorporating peptide spacers between this drug and its carrier (41). These "drug-arm-carrier" conjugates can be specifically cleaved by lysosomal proteases leading to the release of active drug inside target cells. Moreover, these conjugates were more effective than free drug in killing of tumor cells. Similar results were obtained by Trouet *et al.* (16) using oligopeptide spacers varying in length from one to four amino acids. In their studies, the direct conjugate between daunorubicin and carrier was resistant to hydrolysis by lysosomal enzymes and drug was not released intracellularly in an active form. Hydrolysis by lysosomal enzymes and subsequent drug activity was, however, increased following introduction of a tetrapeptide spacer. The tetrapeptide conjugates remained stable in the presence of serum, as required for authentic lysosomotropic drug-carrier complexes, and the chemotherapeutic activity of daunorubicin increased with the carrier's sensitivity to lysosomal hydrolysis. Similar augmentation in the therapeutic index of primaquine was observed in both extraerythrocytic and hepatic stages of murine malaria following linkage of this antimalarial agent to a hepatocyte-targeted glycoprotein via a tetrapeptide spacer (42).

A.1.2.1 Glycoprotein targeting of antivirals

Fiume *et al.* (43) have demonstrated that galactosylated serum albumin conjugated to adenine arabinoside (ara-a) and asialofetuin-ara-a conjugates, administered to mice with ectromelia virus-induced hepatitis, were equally effective in inhibiting virus DNA synthesis in liver without producing significant inhibition of cellular DNA synthesis in intestine and bone marrow. Similar results were observed with ara-c and trifluorothymidine (35-38,44). These findings were, in part, due to selective hepatocyte uptake which in turn reduced the whole animal dosage required for a therapeutic effect (*i.e*, ten-times less conjugated ara-a was required to inhibit viral DNA synthesis as compared to free ara-a). Galactosylated serum albumin has a significant advantage over asialofetuin as a hepatotropic carrier since conjugates prepared with homologous albumin are not immunogenic (43).

A.1.2.2 Glycoprotein targeting of immunostimulants

A number of synthetic immunostimulants are active *in vitro*, however, due to pharmacological problems, their *in vivo* activity is minimized. One approach to overcome this problem has been to conjugate immunostimulants to glycoproteins or neoglycoproteins. For example, Monsigny *et al.* (45) and Roche *et al.* (46) have shown that MDP-conjugated mannosylated neoglycoproteins can be targeted to alveolar macrophages. These macrophages become tumoricidal and mice and rats are protected against metastatic growth of Lewis lung carcinoma. MDP-conjugated mannosylated neoglycoprotein are several orders of magnitude more effective than free MDP in activating macrophages and protecting animals. Recently, Monsigny has conjugated MTP to polylysine partially substituted with gluconyl groups. These conjugates are even better than the neoglycoprotein conjugates in potentiating macrophage activity and are currently in phase I clinical trials in France (see Methods section below).

A.2 Combination antiviral therapy

Experience with anticancer chemotherapeutic agents has clearly demonstrated that combination therapy is often more effective than the additive effects of individual agents. This is particularly apparent when the mechanisms of action of drugs are different. As the number of antiviral agents increases, it is reasonable to believe that similar combination therapy will be effective in viral diseases. Combination therapy offers a distinct advantage over single agent therapy in that the therapeutic dose can be reduced and toxicity minimized. Moreover, the number of potential failures or recurrences due to the selection and overgrowth of drug resistant virus mutants can be limited. A number of observations on antiviral drug combinations with either additive or synergistic effects have been reported (47-55). For example, Fischer *et al.* have demonstrated the *in vitro* synergistic effects of 5'-amino-thymidine and iododeoxyuridine against herpes simplex virus (49). Ayisi *et al.* have shown that combinations of 5-methoxymethyl-deoxyuridine with either vidarabine or phosphonoacetic acid are synergistic against herpesviruses in cell culture (48). In addition, vidarabine in combination with phosphonoacetic acid has been found to produce a synergistic response against herpesviruses and retroviruses *in vitro* (51) and against herpesviruses *in vivo* (52). Combinations of acyclovir with either vidarabine, vidarabine monophosphate or phosphonoformic acid have been reported to produce additive antiviral effects both *in vitro* and *in vivo* (53,54). Similar enhancement of activity has been achieved with combinations of antiviral agents against human influenza viruses *in vitro* (50). The combination of ribavirin with either amantadine or rimantadine hydrochloride resulted in a significantly enhanced antiviral effect against a several subtypes

of influenza A viruses. Recently, combination therapy with azidothymidine and recombinant interferon alpha resulted in synergistic inhibition of human immunodeficiency virus replication (55).

B. OBJECTIVES AND APPROACH

B.1 Phase I studies

B.1.1 Broad spectrum activity of liposome-encapsulated drugs

Our previous studies have demonstrated the effectiveness of liposomes as carriers of antiviral and immunostimulating drugs in the treatment of herpes- and arenavirus infections. The general acceptance of liposomes as carriers of antiviral and immunostimulating drugs will depend, in part, on the demonstration of their effectiveness in a variety of viral diseases. Thus, we will extend our previous DOD contract studies to include additional murine models of diseases of interest to the military, *i.e.*, the bunyavirus, Punta Toro, and the flavivirus, Banzi. We will also study murine models of retrovirus-induced immunosuppression (e.g. Rauscher leukemia and LP-BM5 virus-induced murine AIDS). These viruses were selected because of their different tissue-tropisms (*i.e.*, Punta Toro for liver, Banzi for brain and Rauscher and MAIDS for lymphoid cells). In addition, Punta Toro and Banzi viruses result in acute infections whereas Rauscher and LP-BM5 viruses result in chronic persistent disease.

The effects of chemotherapeutic and immunotherapeutic agents will be assessed by measuring mean survival time, viremia, and/or virus replication in selected organs. Survivors will be examined for virus specific serum antibody and resistance to virus rechallenge. The Punta Toro and Banzi virus models are currently in use in our laboratory. Rauscher leukemia and LP-BM5 viruses have been used as models for screening of antivirals effective against AIDS, a disease of worldwide significance. Studies on these viruses have been conducted in collaboration with Dr. Erik De Clercq of the Rega Institute, Leuven, Belgium. One of us (JDG) spent part of his sabbatical (1987-1988) in Dr. De Clercq's laboratory during which time he gained experience with several retroviral models of immunosuppression. Both the murine AIDS and Rauscher leukemia models are now established in our laboratory.

Our initial studies have focused on ribavirin and MTP-PE since these drugs were effective in our other models. However, subsequent studies will include additional antivirals and/or immunostimulants. The selection of these antivirals and

immunostimulants will be made in consultation with the contracting officer.

B.1.2 Selective drug targeting by neoglycoproteins

We have demonstrated in studies supported by previous DOD contracts that liposomes are useful vehicles for the delivery of antivirals and immunostimulants. However, these vehicles have some limitations as targeting agents in that they are compartmentalized primarily by the reticuloendothelial system. Therefore, a second targeting approach has employed conjugation of drugs to neoglycoproteins. This approach has allowed us to deliver drugs to more specific sites of viral infection. Initially, our studies have focused on ribavirin and MTP-PE since these have proven effective following liposome encapsulation. Subsequent studies will employ additional antivirals and immunostimulants which will be selected in consultation with the contracting officer.

One of our goals for the first year of this contract was to determine the effectiveness of conjugated drug delivery in the treatment of virus infections which occur at different tissue sites. Future studies will compare the relative effectiveness of different targeting approaches using both murine and guinea pig models of viral diseases described in our contract proposal. Successful therapeutic approaches will then be extended to the nonhuman primate models of influenza and Punta Toro previously described.

Homologous serum albumin with added terminal mannose or galactose residues will be conjugated with drugs to target them to macrophages and hepatocytes, respectively. Drug conjugates will be chemically characterized to determine the amount of drug per carrier molecule and biologically characterized to determine functional activity. Conjugation of antivirals with galactosylated albumin should provide a more efficient delivery to infected hepatocytes while conjugation of immunostimulants to mannosylated albumin should enhance the uptake by macrophages and monocytes. Dr. Monsigny from the Universite' d'Orleans, Centre de Biophysique Moleculaire du Centre National de la Recherche Scientifique, Orleans, France, will be collaborating with us in the preparation and characterization of drug-conjugated proteins. Dr. Monsigny has had extensive experience in conjugating drugs such as muramyl dipeptide and tripeptide to neoglycoproteins. In addition, he has recently developed a delivery system consisting of poly-lysine partially substituted with gluconyl groups which is more a more effective carrier than serum albumin. This carrier has recently received approval by the French medical authorities for use in man.

B.1.3 Combination antiviral/immunostimulant therapy

A number of newly developed drugs show promising antiviral activity *in vitro*. However, *in vivo*, many of them cause adverse side effects including damage to components of the immune system. Thus, while antivirals may reduce viral burdens in target organs, a host with a compromised immune system may not effectively remove residual virus. Therefore, any damage to immune components should be compensated for during antiviral chemotherapy. Our approach to this problem will be to attempt to balance the immunosuppressive effects of antivirals with administration of immunostimulants. This strategy has the added advantage that immunostimulants may also act synergistically in those situations where antivirals have minimum or no immunosuppressive effects. Our preliminary data (Contract DAMD 17-84-C-4144 and J. Cell Biology, Supplement 12B, 1988, Abstract W-102 page 255) clearly indicates that combination antiviral/ immunostimulant therapy is indeed effective in the treatment of herpesvirus and arenavirus infections.

Most immunostimulants possess a unique set of immunomodulating features and provide varying degrees of benefit to the infected host depending on the tissue site and degree of virus infection. For example, some immunostimulants activate macrophages and induce high levels of interferon, whereas others activate macrophages but are poor inducers of interferon. Thus, the judicious selection of an immunostimulant to be used in combination therapy with an antiviral requires 1) an understanding of the biological effects of the immunostimulant on the components of the immune system, 2) a knowledge of the mode of action of the antiviral agent, and 3) an understanding of the immune response to the virus infection.

Initially our attention will focus on combination therapy using ribavirin and MTP-PE in murine, guinea pig and primate models of disease since the effects of these drugs have already been characterized in our earlier studies. As other promising antivirals and immunostimulants are developed and characterized under DOD contracts, we will attempt to enhance their efficacy either alone or in combination by targeting using liposomes, or neoglycoproteins. In these studies we will select immunostimulants which have a broad spectrum effect on both specific and nonspecific components of the immune system. Murine, guinea pig and primate models will be used to evaluate therapeutic potential of selected drugs.

B.2 Phase II studies

B.2.1 Preclinical evaluation of liposome-encapsulated ribavirin and MTP-PE

In our previous studies we have shown that encapsulation of ribavirin and/or MTP-PE into liposomes resulted in improved targeting and therapeutic effectiveness when examined in both murine and guinea pig models of viral disease. While these results are encouraging, they do not necessarily ensure the usefulness of liposome-encapsulated drugs in viral diseases of man. A more reliable predictor of clinical usefulness should come from studies performed in nonhuman primates. Thus, in this renewal we propose to study whether targeting of antivirals and/or immunostimulants will enhance the therapeutic efficacy of these drugs in viral diseases of animal species more closely related to man (squirrel and African green monkeys). Since the liver and lung appear to be important target organs in the pathogenesis of several virus infections, we will focus our attention on infections occurring in these organs.

One of the viruses to be examined in these animals will be influenza. In this model animals will be challenged intranasally with virus and examined for fever and virus shedding. Antivirals and immunostimulants will be administered individually or in combination as well as in free or liposome-encapsulated form. Since free ribavirin has been shown to be effective in the therapy of influenza in squirrel monkeys, our studies with this drug will be designed to improve the therapeutic index of this drug via targeted delivery and/or combination with an immunostimulant.

The hepatotropic Punta Toro virus will serve as a second model of viral disease. In this model, African Green monkeys will be challenged with virus and treated with free or liposome-encapsulated antivirals and/or immunostimulants. Animals will be examined for viremia and hematological and liver enzyme changes.

B.2.2 Timing of Phase II studies

Phase I studies will allow us to compare the efficacy of liposomes and neoglycoproteins as targeting vehicles for antivirals and/or immunostimulants in various viral disease models. These models provide us with an opportunity to examine the effectiveness of targeted drug delivery in both acute and chronic virus infections in which the primary sites of replication or dissemination vary. Phase II studies will allow us to test targeting approaches, proven to be effective in Phase I studies, in nonhuman primate models of

viral disease. These studies should enable us to more accurately predict their clinical potential in man.

Our initial studies using liposomes as a targeting vehicle for ribavirin and/or MTP-PE have already proven successful in several of the murine and guinea pig models of disease. Thus, we conducted studies under Phase II studies using liposome-encapsulated ribavirin and/or MTP-PE immediately. When other targeting vehicles and/or drugs proved successful in Phase I studies they were then be tested in Phase II studies. In this way Phase I and Phase II studies were conducted concurrently throughout the contract period.

C. RESULTS

C.1 YEAR ONE OF CONTRACT

The first year of this contract was devoted to the establishment of new animal models for drug evaluation and to the production of sufficient amounts of conjugated antivirals and immunomodulators which can be tested in *in vivo* models of viral disease. More specifically, the initial year of this contract has focused on the following:

1). Development of the LP-BM5 (Murine AIDS) virus model.

2). Development of an *in vitro* viral cytopathic assay for evaluation of antivirals and immunomodulators in human macrophages.

3). Establishment of a dengue virus squirrel monkey model for the evaluation of antiviral agents.

4). Use of squirrel monkeys as a model animal for the evaluation of immunomodulating agents.

5). Antiviral profile of a new recombinant human interferon hybrid (rhuIFN-alpha B/D), from CIBA-GEIGY.

6). Conjugation of MDP, ribavirin and phosphonylmethoxyethyl adenine (PMEA) to mannosylated bovine serum albumin or polylysine carriers.

7). Evaluation of the therapeutic value of MDP-conjugates in murine models of viral pneumonitis and hepatitis.

8). Immunostimulating properties of MDP-BSA-Mannose and MDP-Polylysine-Mannose: Effects on RES function, macrophage phagocytosis and cytotoxicity.

9). Evaluation of the antiviral potential of ribavirin and PMEA polylysine carriers in virally infected human macrophages.

10). Evaluation of the therapeutic value of PMEA-conjugates in a murine model of pneumonitis.

C.1.1 Murine AIDS model

Together with CIBA-GEIGY scientists, we have developed a method to quantitatively assess lymphadenopathy and splenomegaly using nuclear magnetic resonance (NMR) imaging. The advantage of this technique is that mice do not have to be sacrificed and can be monitored over the entire course of the disease. An example of the NMR images produced by this method is shown in Figure 1. Note the clarity of parathymic, axial and inguinal lymph nodes and spleen. Automated analysis of these structures provides the means with which node and spleen size of individual animals can be objectively determined over a prolonged period of therapy.

In addition to NMR analysis of internal organs, we have also developed an *in vivo* virus challenge model in which immunosuppressed mice become susceptible to lethal infection with HSV-1. This has been a very valuable adjunct measurement of the degree of suppression and has allowed us to gain further insight to the value of antiviral agents to slow or prevent immunosuppression from occurring. Table 1 illustrates the effects of HSV-1 superinfection on LP-BM5 immunosuppressed mice. This enhanced susceptibility provides an opportunity to study therapeutic intervention in a murine model of retroviral-induced immunosuppression as well as on HSV-1 superinfection. Another feature of this model which has important implications is our discovery that adherent peritoneal cells obtained from LP-BM5 virus infected mice are able to transmit infection. This indicates that macrophages are infected at some time during the course of this disease (Table 2).

The effectiveness of experimental anti-herpes and retroviral agents administered to immunosuppressed mice prior to and during HSV-1 superinfection has been examined. This data is presented in the manuscript entitled "9-(2-Phosphonylmethoxyethyl) Adenine (PMEA) in the Treatment of Murine Acquired Immunodeficiency Disease (MAIDS) and Opportunistic Herpes Simplex Virus Infections" which has been included in the Appendix. It should be noted that some of the MAIDS developmental studies were performed while one of us (JDG) was working in Basel, Switzerland during the first year agreement of our contract.

C.1.2 Human monocyte antiviral assay

Development of an *in vitro* assay for the evaluation of antivirals and immunomodulators which have been chemically modified (ie. conjugated to glycoproteins or encapsulated into liposomes) is needed to ensure that the biological activity of these drugs has not been reduced or destroyed. Moreover, it is important that cells used in the *in vitro* assay system are relevant to the targeting approach which we are using. A second manuscript, "Use of Human Monocytes for the Evaluation of Antiviral Drugs: Quantitation of HSV-1 Cytopathic Effects" describes this procedure and is included in the Appendix of this report. This manuscript provides a through description of the methodology used to assay antivirals and immunomodulators in cultured human macrophages. Several drugs, including Poly I:C-LC, rhuIFN-alpha B/D, acyclovir and ribavirin have been evaluated in this assay (See Appendix). Poly I:C-LC, rhuIFN-alpha B/D and acyclovir were highly effective in protecting macrophages from the cytopathic effects of HSV-1 infection. In contrast, ribavirin had little or no effect. We are currently examining the ability of other viruses such as Pichinde (AN 3739), Punta Toro, Sandfly Fever and Influenza (Aichi) viruses to cause a cytopathic effect in these macrophages. If successful, these will be incorporated into our antiviral assays.

C.1.3 Development of a dengue virus squirrel monkey model

A primate adapted dengue type 1 virus (West Pac 74) was obtained from Dr. Ken Eckles (WRAIR Wash. DC) and used to infect squirrel monkeys, *Saimiri sciureus*. This virus was supplied in lyophilized form and was reconstituted in pyrogen free water prior to intramuscular inoculation (0.5 ml, approximately 5 X 10^4PFU) into the upper forearm. Dengue virus was inoculated into male *S. sciureus* monkeys which were screened for the presence of neutralizing anti-dengue antibodies by Dr. Ken Eckles. Animals were examined daily for viremia, fever, liver enzyme and differential white blood cell counts. Preliminary data are now being evaluated and we hope to have this dengue primate model established by the end of this summer.

C.1.4 Use of squirrel monkeys as a model for evaluation of immunomodulating agents

Squirrel monkeys were held in our animal quarters for several days prior to being bled to determine baseline serum interferon levels. These monkeys were gavaged with 25 mg/kg of CL 246,738 and their serum collected each day for three days. Gavage (25 mg/kg) was

repeated on day four and serum samples collected on days five and seven. All serum samples were analyzed for interferon levels in primary human foreskin cells and VSV as the challenge virus. Cell destruction was evaluated using the neutral red dye uptake and the colorimetric assay described in the attached manuscript (See Appendix) . Briefly, serial (three-fold) dilutions of serum were added to foreskin monolayers in microtiter plates and the plates were incubated for 18 hours. Treated cells were challenged with VSV and incubated for another 48 hours prior to the addition of neutral red. Note that in Table 3, the dilution of the NIH international human interferon standard (10,000 units by plaque reduction) was 4706 by this assay.

Three monkeys were examined for serum interferon levels. All had maximal responses at day two post inoculation; monkey three had the highest interferon response (260 U/ml = 520 IU/ml) and monkey two the lowest (78 U/ml). Unexpectedly, none of the three responded to a second gavage (CL 246,738, 25 mg/kg) administered four days after the first.

C.1.5 Antiviral profile of rhuIFN-alpha B/D

RhuIFN-alpha B/D is a human hybrid interferon which is cross-species active and can be utilized in a variety of animals. Figure 2 illustrates the potent antiviral activity of rhuIFN-alpha B/D compared to alpha A (Roferon) or gamma interferons in human monocytes infected with HSV-1. Note the lack of gamma activity and potent rhuIFN-alpha B/D response.

The *in vivo* antiviral activity of rhuIFN-alpha B/D in murine models of HSV-1 pneumonitis (VR3) and encephalitis (MacIntyre) are presented in the manuscript entitled "Antiviral activity of a human recombinant interferon alpha B/D hybrid" (Appendix). The results from these studies are quite impressive considering that treatment was initiated several hours after infection. Likewise, similar efficacy was demonstrated in the Friend murine leukemia and LP-BM5 models. Note that rhuIFN-alpha B/D was effective in slowing the onset of virus-induced immunosuppression (as measured by mitogen response), splenomegaly and lymphadenopathy when administered subcutaneously every second day (50 million units/kg) for 14 days following infection. Moreover, when administered together with suboptimal doses of AZT, rhuIFN-alpha B/D was able to augment the effects of AZT. Similar augmentation was observed when other nucleoside analogues were used together with rhuIFN-alpha B/D (See Appendix).

C.1.6 MDP and ribavirin conjugates

The procedures used to conjugate antivirals and immunopotentiators to BSA or Poly-L-lysine are as follows. In the first step, mannosylated BSA was prepared by allowing a-D-mannosyl-pyranosyl-phenyl-isothiocyanate (MPPI) to react with BSA according to a method derived from McBroom, Samanen and Goldstein (Meth. Enzymol., 28:212, 1972). Briefly, 301.5 mg (1 mmol) of p-nitrophenyl-a-D-manno- pyranoside (Sigma) was dissolved in 20 ml of 4:1 methanol:water mixture. To this solution was added 30 mg of 10% Palladium on charcoal (Merk). The suspension was stirred under hydrogen (1 atm) at room temperature for two hours and then filtered. The filtrate was evaporated under reduced pressure at 40 C. The p-nitrophenyl- a-D-manno-pyranoside thus obtained was dissolved in 30 ml of 0.1 M sodium carbonate, pH 8.6. To this solution was added 30 ml of chloroform containing 2 mmoles of thiophosgene. This mixture was shaken for 30 minutes and the a-D-manno-pyrannosyl-phenyl- isothiocyanate thus formed was crystallized. The crystals were washed in chloroform and cold distilled water. 31 mg of recrystallized MPPI was added to 25 ml of a solution containing 268 mg BSA in 0.1 M sodium carbonate (pH 9.5). After 20 hour stirring , the resulting manno-pyrannosyl-thiocarbamyl serum albumin (Man-BSA) was purified by gel filtration on an Ultrogel-202 column in distilled water. The mannose content of Man-BSA was determined by the resorcinol-sulfuric acid method.

MDP serum albumin conjugate was prepared by adding MDP hydroxysuccinimide ester to a solution of BSA or Man-BSA in 1 M sodium bicarbonate buffer, pH 8.5. MDP-hydroxysuccinimide ester was prepared by dissolving 100 mg (0.2 mmole) MDP, 46 mg (0.24 mmole) dicyclohexyl- carbodiimide and 26.6 mg (0.24 mmole) N-hydroxysuccinimide in 2 ml of freshly distilled dimethyl formamide. After 24 hours at 25° C, MDP was quantitatively converted into MDP-hydroxysuccinimide ester. The solution of MDP-hydroxysuccinimide ester in dimethylformamide was added to 17 ml solution of 170 mg of Man-BSA or BSA in 1 M sodium bicarbonate buffer at pH 8.5. The reaction mixture was stirred for 24 hours at room temperature. The conjugate was purified by gel filtration on Ultrogel 202 column in phosphate buffered saline, pH 7.4. The MDP content of the conjugate was determined by the method of Levy and McAllan (Biochem. J., 73:127, 1959). Similar procedures were used in the conjugation of ribavirin to Poly-L-lysine and BSA.

Table 4 lists the MDP and Ribavirin BSA and Poly-L-Lysine conjugates prepared by Dr. Michel Monsigny in Orleans, France and currently under study in our laboratory. Four of these conjugates (ie. MMB 350, MMB 351, PLL Mannose, and MDP-PLL-Mannose) have been studied in detail (see below). A structural drawing of the MDP-Poly-L-Lysine- Mannose (MDP-PLL-Mannose) and

Ribavirin-PLL-Mannose conjugates is presented in Figure 3. Note that free amino groups are blocked with carbohydrate groups to prevent nonspecific cellular attachment and rapid clearance from the circulation. The MDP linkage illustrated in Figure 3 is highly stable and has been successfully employed by Dr. Monsigny for a number of years. Several variations in the chemical linkage of Ribavirin to poly-L-lysine (*e.g.* the addition of glycyl-glycine spacers) are under investigation in Dr. Monsigny's lab. These studies will enable us to select the optimal drug linkage for stability and biological activity.

C.1.7 MDP conjugates in murine models of HSV-1 induced pneumonitis and hepatitis

Figure 4A illustrates the therapeutic activity of MDP-BSA conjugates in a murine model of herpesvirus (HSV-1) hepatitis. In this infection model, virus is administered intravenously and within 2-3 days results in viremia and fulminant liver infection. Note that when drug was administered intravenously (10 μg/mouse, 2 days prior to infection, on the day of and two days following infection), MDP-BSA-Man-6-PO_4 (MMB 351) was highly effective in preventing death and in enhancing mean survival times. A similar effect was observed with MDP-BSA (MMB 350) (data not shown). MDP-BSA-Man-6-PO_4 (administered i.v., 10 μg/mouse as above) was more effective than free MDP in prolonging the mean survival time of mice infected intranasally with a strain of HSV-1 causing pneumonitis (Figure 5A).

Figure 4B illustrates the therapeutic activity of MDP-Poly-L-lysine-Mannose in the murine model of HSV-1 hepatitis. Note that intravenous administration of drug (10 μg/mouse) 2 days prior to infection, on the day of infection, and 2 days following infection resulted in 40% survival and prolonged the mean survival time of mice. A significant prolongation of mean survival time was also observed in mice receiving intravenous MDP-Polylysine-Mannose (10 μg/mouse) and infected intranasally with HSV-1 (Figure 5B).

C.1.8 Immunostimulating properties of MDP conjugates

C.1.8.1 Stimulation of RES functions

Mice treated intravenously with 10 ug of MDP-poly L lysine two days prior to the intravenous inoculation of chromium-labeled sheep red blood cells were able to clear SRBC from their circulation at a much greater rate than mice receiving carrier alone or free MDP (Figure 6).

C.1.8.2 Effects on macrophage phagocytosis and cytotoxicity

No significant differences in peritoneal macrophage phagocytosis was observed 2 days following a single intraperitoneal inoculation of MDP-BSA or MDP-BSA-Man-6-PO_4 (10 μg/mouse) (Table 5). This negative response may be due to drug dosage and/or time of administration; therefore, future experiments will examine these parameters.

Little change in macrophage cytotoxicity was observed 2 days following a single inoculation with either the BSA or Polylysine MDP conjugates (10 μg/mouse) (Tables 6 & 7). Nonetheless, there was a suggestion that cytotoxicity levels were elevated (see 20 and 10:1 effector to target cell ratios in Table 6) following administration of either MMB 350 (MDP-BSA) or MMB 351 (MDP-BSA-Man-6-PO_4). Future experiments will examine macrophage cytotoxicity levels at later times (*i.e.* 3 or 4 days post inoculation) following either single or multiple drug dosages.

C.1.9 Evaluation of the antiviral activity of nucleoside analogue poly-lysine conjugates in human macrophages

We now have several nucleoside analogues (e.g. PMEA, ribavirin and AZT) conjugated to poly-lysine. Preliminary data indicate that PMEA conjugated to poly-lysine was as good or better than free PMEA in preventing the lysis of human macrophages infected with HSV-1 (Figure 7). Free or conjugated ribavirin were not effective in preventing the cytopathic effects of HSV-1 in human macrophages. Future studies will employ models of RNA virus infections (Influenza, Pichinde, and Punta Toro) which may be more susceptible to the antiviral activity of ribavirin.

C.1.10 Evaluation of PMEA-conjugates in a murine model of pneumonitis

As illustrated in Table 8 the therapeutic activity of the PMEA-poly-L-lysine conjugate was 8-10 times better than free PMEA in reducing virus titers in lungs of mice infected with HSV-1. In this model, four week old mice are infected intranasally with 1 x 10^5 pfu and then treated several hours later and on day 2 and 4 post infection with either free or conjugated drug. Lungs were removed on day four and a 10% homogenate assayed for infectious virus.

C.2 YEAR TWO OF CONTRACT

The second year of this contract was devoted to studies aimed at further characterizing the immunological profiles of three drugs, Poly I:C-LC, CL 246738 and Riker-3M. These studies were designed to examine the possible detrimental effects of multiple drug dosage, using drugs which have proven to be effective in our primary screen when given in a single dose. These studies were prompted by the concern that multiple dosages of a drug may result in acute toxicity. Therefore, it was decided that experiments aimed at evaluating the effects of varying dosages of an immunomodulator on resistance to viral infection and changes in immunological functions should be planned. In particular, the effects of multiple drug dosages on resistance to Banzi virus challenge, interferon induction, RES stimulation, and NK cytotoxicity were examined. We have also evaluated the carrier potential of liposomes in the delivery of immunomodulating agents which will soon enter clinical trials at USAMRIID. Since Poly I:C-LC is a prime candidate for initial clinical trials, we performed a series of experiments to characterize the therapeutic advantage of liposome delivery of this drug.

Studies performed in the second year of this contract have also included 1) the development of a Dengue 2 virus model, 2) the evaluation of interferon responses in primates treated with free and liposome encapsulated Poly I:C, 3) a comparison of the efficacy of Poly I:C-LC and dextran-Poly I:C conjugates, and 4) an evaluation of liposome encapsulated AVS-5587. In addition, our subcontractor, Dr. Monsigny, has prepared AVS-5587 conjugated to mannosylated poly-L-lysine for evaluation in macrophage functional studies and in *in vivo* resistance.

C.2.1 Evaluation of Poly I:C-LC, Riker-3M and CL 246738

C.2.1.1 Serum Interferon

Table 9 illustrates the serum interferon levels in mice receiving oral doses (4 mg/ms) of Cl 246738 every 3 days for 12 days. Note that one day following the first gavage, mice had significant levels of circulating interferon in their serum. When examined again one day after each of their subsequent gavages, the interferon levels were only slightly elevated suggesting that multiple drug treatment may result in a hyporesponsive state. It is appreciated that this result was obtained using a single dose (recommended by the Project Officer) which induces the maximum degree of macrophage cytotoxicity (56) although lower doses are optimal for NK cell activation and interferon induction (57). It is possible that the suppressive acitivity of the drug in multiple doses was due to the super-optimal dose used in these experiments. Similar results were observed with the Riker 3-M compound, R837

(Table 10). It should be pointed out that these results were obtained using a single dose of the drug and a single point of serum sampling. It has been reported that in guinea pigs receiving 3 mg/kg of this drug, maximum level of serum interferon is observed at 12 hours after treatment although significant levels persist at 24 and 72 hours (58). It is likely that 24 hours post treatment is not the optimal time in mice and may explain the very low levels of interferon (comparable to control levels) in mice receiving multiples doses of the drug.

C.2.1.2 Natural Killer Cell Cytotoxicity

Mice were divided into 4 groups. Group 1 received a single gavage with Riker-3M (10 mg/kg) 2 days prior to evaluation of NK cytotoxicity. Group 2 received gavage on days 2 and 4; Group 3 on days 2, 4, and 6; and Group 4 on days 2, 4, 6 and 8. As illustrated in Table 11, all groups showed significantly elevated NK cytotoxicity at all effector to target cell ratios. However, multiple doses appeared to result in a slight diminution of NK cell activity. Thus, while multiple doses of Riker-3M did not grossly effect NK cell activity they may have had some slight adverse effects.

A similar protocol was used with CL 246738. Similar to our observations with Riker-3M, multiple doses appeared to enhance NK cytotoxicity; however, a slight diminution of this activity occurred following multiple doses (Table 12).

C.2.1.3 Macrophage cytotoxicity

Mice were divided into 4 groups. Group 1 received a single gavage with Riker-3M compound (10 mg/kg) 2 days prior to evaluation of macrophage cytotoxicity. Group 2 received gavage on days 2 and 4; Group 3 on days 2, 4, and 6; and Group 4 on days 2, 4, 6 and 8. An additional group of mice was injected with *Propionibacterium acnes* (70 mg/kg) as a positive control. As illustrated in Table 13 no significant macrophage cytotoxicity was observed in any of the groups. Macrophages from mice injected with *P. acnes* exhibited the expected anti-tumor cytotoxic activity (data not shown). Similar results were obtained with CL 246738 (Table 14).

C.2.1.4 Effects on the reticuloendothelial system

The data in Table 15, shows that while one dose of CL 246738 was stimulatory, two and three doses of the drug had a somewhat reduced effect of RES activity. Surprisingly, four doses also was

stimulatory. Currently we have no explanation for this anomaly. Similar results were obtained with Riker-3M (Table 16).

C.2.1.5 Phagocytosis

Mice were divided into 4 groups. Group 1 received a single gavage with Riker-3M (10 mg/kg) 2 days prior to evaluation of phagocytic activity of peritoneal cells. Group 2 received gavage on days 2 and 4; Group 3 on days 2, 4, and 6; and Group 4 on days 2, 4, 6 and 8. As illustrated in Figure 8 when drug was administered two days prior to assay, a significant enhancement in phagocytic activity was observed. However, multiple drug doses did not result in sustained phagocytic activity (Figures 9 - 11).

C.2.1.6 Resistance to Banzi virus following multiple injections with Riker-3M or CL 246738

Mice were divided into 5 groups. Group 1 received a single gavage with Riker-3M (10 mg/kg) two days prior to virus challenge; Group 2 received gavage 4 days prior to challenge; Group 3 received gavage 6 days prior to challenge; Group 4 received gavage 8 days prior to challenge; and Group 5 received gavage 10 days prior to challenge. Figure 12 illustrates that animals in Group 1 were able to resist virus lethality (60% protection) while animals in Group 2 were only marginally protected (30% protection). None of the other three groups were protected.

A similar protocol was used for CL 246738. Except for animals which received drug 10 days prior to virus challenge (Group 5) significant protection was observed in each of the other groups (Figure 13). Note the time dependency of drug administration and level of protection. Moreover, animals treated with CL 246738 were more resistant than those treated with Riker-3M.

C.2.1.7 Resistance to Banzi virus following multiple injections with Poly I:C-LC

Mice were divided into 11 groups in which the following treatment protocols were examined. These treatment protocols were selected to complement ongoing studies at Ft. Detrick under the direction of Dr. Meir Kende.

Group	Day of Poly I:C-LC Injection (i.v. 1 mg/kg)
A.	-20, -15, -10, -5, 0, +5, +10 (saline i.v. control)
B.	-20, -15, -10, -5, 0, +5, +10
C.	-10, -5, 0, +5, +10
D.	-11, -6, -1,+4, +9
E.	-12, -7, -2,+3, +8
F.	-13, -8, -3, +2, +7
G.	-14, -9, -4, +1, +6
H.	-21, -16, -11, -6, -1, +4, +9
I.	-22, -17, -12, -7, -2, +3, +8
J.	-23, -18, -13, -8, -3, +2, +7
K.	-24, -19, -14, -9, -4, +1, +6

As illustrated in Figures 14a and 14b, groups B, D, E, G, H, and K were able to resist challenge with Banzi virus. In contrast groups F (Figure 14a), J, and I (Figure 14b) were not protected and mice died at about the same time as saline controls. We have no explanation as to why mice in groups F, J, and I were still fully susceptible to Banzi infection especially since their therapy differed by only one day (compare group F with groups E and G in Figure 14a, and groups I and J with groups H and K in Figure 14b). In general, however, it appears that multiple dosages have no deleterious effects on resistance to Banzi virus infection.

C.2.2 Evaluation of Dengue 1 virus (West Pac) infection of squirrel monkeys

Dengue 1 virus infection in squirrel monkeys, which were confirmed to be seronegative prior to infection was evaluated. Monkeys were bled prior to virus infection and serum samples were evaluated for the presence of neutralizing antibody. None of the animals had detectible antibody levels. These animals were inoculated intramuscularly (0.5 ml) into their forearms with an undiluted lyophilized preparation of Dengue 1 (West Pac 74) containing 1 X 10^5 plaque forming units per ml. Fever was monitored and serum samples collected daily from each monkey for 12 days. In addition, differential counts and liver enzyme changes were evaluated. As illustrated in Table 17, no change in temperature or liver enzymes were observed at any time following infection and only a slight viremia was evident. This viremia was slightly higher at day 5 than at day 4.

C.2.3 Targeting of Poly I:C and 7-thia-8-oxoguanosine (AVS-5587)

C.2.3.1 Liposome encapsulation of Poly I:C and AVS-5587

These experiments were designed to evaluate the effect of administering the immunomodulator Poly I:C or the antiviral AVS-5587 in a carrier which should release the drugs slowly and hence sustain their concentration in the body (circulation) for a longer period. Also, encapsulation should protect the drug from the detoxifying effects of host enzymes. These drugs were administered intravenously to achieve the maximum blood level quickly. As illustrated in Table 18, the encapsulation efficiency of Poly I:C was quite good. Thus, one hour after preparation, 50% of the drug could be shown to be associated with liposomes. Moreover, this association was stable over a 36 hour period as illustrated by the fact that 40% was still liposome-associated. Encapsulation of AVS-5587 was not as good as that observed for Poly I:C; however, 30% of the drug was liposome associated at one hour and 20% at 36 hours following the initial preparation. The data suggest that both Poly I:C and AVS-5587 can be easily incorporated into freeze dried liposomes made from synthetic lipids (CIBA-GEIGY). Liposomes loaded with these drugs are stable over a 36 hour period.

C.2.3.2 Interferon induction by free and liposome-encapsulated Poly I:C

Table 19 illustrates the serum interferon titers in mice inoculated intravenously with either free or liposome-encapsulated Poly I:C at eight hours post-injection. Note that liposome-encapsulated Poly I:C (589 IU) was as good as free Poly I:C (437 IU) in inducing interferon. Moreover, the kinetics of interferon response revealed that liposome-encapsulated Poly I:C was as good or even slightly better than free Poly I:C when examined at 12 and 24 hours post inoculation (Table 20).

C.2.3.3 Natural killer cell activity following administration of free or liposome-encapsulated Poly I:C

Natural killer cell activity was examined at 1, 2 and 3 days following the intravenous administration of drug (Table 21). Note that both free and liposome-encapsulated Poly I:C (50 μg/kg) were good enhancers of NK activity on any of the three days examined and no significant difference between the two drug formulations was

observed. In addition, both appeared to have maximal effects at two days post infection.

C.2.3.4 Effects of liposome encapsulation of Poly I:C on reticuloendothelial system function

Table 22 represents a composite of 3 clearance experiments. As illustrated, liposome-encapsulation significantly enhanced Poly I:C's ability to stimulate the reticuloendothelial system as measured by the increased rate of labelled SRBC clearance (T/2). This effect was demonstrated at dosages of 500, 50 and 5 μg/kg.

C.2.3.5 Effects of liposome encapsulation of Poly I:C on macrophage cytotoxicity

Table 23 illustrates the cytotoxic potential of macrophages stimulated with free or liposome-encapsulated Poly I:C. Note that our results are variable; nonetheless, a positive effect for liposome-encapsulated drug was observed on at least one occasion (exp. #3; cytotoxicity = 18 for free and 82% for encapsulated drug). A second experiment (#2) also indicated a positive effect, but the sham liposome effect invalidated this experiment.

C.2.3.6 Effects of free or liposome-encapsulated Poly I:C on splenic lymphocyte subpopulations

No significant differences in splenic cell populations were noted following the administration of either liposome-encapsulated or free Poly I:C (Table 24).

C.2.3.7 Effects free or liposome-encapsulated Poly I:C on phagocytic activity of peritoneal macrophages

A number of experiments were performed to analyze the phagocytic activity of PEC from Poly I:C treated mice. As illustrated in Figures 15 and 16, a slight increase in phagocytic activity was sometimes observed following treatment with liposome versus free Poly I:C. However, this effect was not always seen (Figures 17 and 18). Thus, liposome-encapsulated Poly I:C at the dose examined had little, if any, effect on phagocytosis by peritoneal macrophages.

C.2.3.8 Protection against Banzi virus infection following administration of liposome-encapsulated Poly I:C

Figures 19-22 illustrate the protection afforded mice following Poly I:C administration. Note that liposome-encapsulated drug was as effective and in some instances more effective than free drug in protecting mice from lethal Banzi virus challenge. Quite surprisingly, as little as 5 or 0.5 μg/kg was effective.

C.2.3.9 Interferon induction by free or liposome-encapsulated Poly I:C in primates

In an effort to determine whether selective delivery of Poly I:C to macrophages would enhance the interferon response, we inoculated squirrel monkeys intravenously with 0.2 mg/kg free or liposome encapsulated Poly I:C. One day following inoculation animals were bled and serum interferon levels were determined by a neutral red dye exclusion assay.

Table 25 illustrates the serum interferon levels 24 hours after drug administration. As indicated, no interferon was observed in pre-bleed samples and none was induced following administration of empty liposomes (Group 1, sham). In contrast, liposome encapsulated Poly I:C was a potent interferon inducer (Group 4). The encapsulated drug (Group 4) was even better than free Poly I:C (Group 2) or the Poly I:C-LC derivative (Group 3). Similar results were obtained in both experiments 1 and 2.

Monkeys were also inoculated with Poly I:C-dextran which was obtained from Dr. Bellows. At 24 hours following intravenous administration, a significant serum interferon response was observed (Table 25, Group 5). This response was greater than that observed with free Poly I:C or Poly I:C-LC but was not as great as that observed with liposome encapsulated Poly I:C (compare Group 5 with Groups 2,3, and 4 in Table 25).

C.2.3.10 Interferon response to free or liposome encapsulated AVS-55587

Groups of three mice were inoculated, intravenously, with 10 mg/kg AVS-5587 either in a free or liposome encapsulated form. This was the highest dose of drug which could be administered due to solubility problems. Mice were bled at 4, 12, and 24 hours post inoculation and examined for serum interferon. Only low levels of interferon were observed at 4 and 12 hours post administration and no differences were noted between free and encapsulated drug (Table 26).

C.2.3.11 Stimulation of the reticuloendothelial system by free or liposome encapsulated AVS-5587

As illustrated in Table 27, neither free nor liposome encapsulated AVS-5587 (10 mg/kg) stimulated the reticuloendothelial system as measured by the clearance rate of ($T_{\frac{1}{2}}$) injected labeled SRBC from the circulation.

C.2.3.12 Macrophage cytotoxicity following administration of free or liposome-encapsulated AVS-5587

Table 28 illustrates the lack of macrophage cytotoxicity following intraperitoneal inoculation of 50 mg/kg of AVS-5587. A higher concentration of drug was used this experiment due to the fact that we were able to inoculate mice with a larger volume intraperitoneally. Note that neither free nor liposome encapsulated drug was able to render peritoneal macrophages cytotoxic. In contrast *P. acnes* was highly effective.

C.2.3.13 Peritoneal exudate cell phagocytosis following treatment with free or liposome-encapsulated AVS-5587

Figures 23 and 24 illustrate the phagocytic activity of peritoneal exudate cells from control mice and mice treated with AVS-5587. As illustrated in both figures neither oral nor intraperitoneal administration of drug had any effect on phagocytic activity of peritoneal cells. The oral route was employed because of published reports indicating that this route was effect in resistance to virus infection.

We also tested whether liposome encapsulation might increase the ability of AVS-5587 to augment peritoneal cell phagocytosis. Note that liposome encapsulated drug given 2 days before testing had no effect on drug activity (Figure 25). The lack of an effect was not due to the dose employed since increasing drug concentration five fold had no effect. Similar results were obtained with free or encapsulated drug (Figures 26 and 27).

C.2.3.14 Effects of free or liposome-encapsulated AVS-5587 on lymphocyte subpopulations

Table 29 indicates that AVS-5587 had no effect on the proportion of B and T cells in the spleen or the absolute numbers of these cells. We are uncertain as to the biological significance of the

apparent splenomegaly which occurred following administration of the $NaHCO_3$ vehicle.

C.2.3.15 Effects free or liposome-encapsulated AVS-5587 on NK cytotoxicity

Table 30 illustrates the inability of AVS-5587 to augment splenic NK cytotoxicity. Note that neither free nor liposome encapsulated drug was effective at any of the three Effector:Target cell ratios employed.

C.2.3.16 Resistance to Banzi virus following administration of free or liposome-encapsulated AVS-5587

While the *ex vivo* interferon and immunological function studies described above suggest a lack of immunostimulatory activity by AVS-5587, some enhancement of resistance to Banzi virus infection was observed (Figures 28 and 29). Note that while both free and liposome encapsulated drug were equally effective in preventing death when administered one day before infection (Figure 28), liposome-encapsulated drug was slightly better when administered on the day of infection (Figure 29). In addition, both free and encapsulated drug were equally effective in prolonging mean survival times when given one day before infection (Figure 28).

C.2.4 Dengue 2 infection in squirrel monkeys

Since our initial attempts to develop a dengue squirrel monkey model using the Type 1 dengue virus (West Pac) provided by Dr. Ken Eccles (WRAIR) were unsuccessful, we initiated studies using a Type 2 dengue virus (strain 16681) provided by Dr. T. Monath. Monkeys were screened for anti-dengue antibodies prior to being included in this study. Animals were examined daily for five days prior to virus inoculation for baseline values for fever and white blood cell counts. Monkeys were inoculated subcutaneously with Dengue 2 (1×10^6 p.f.u.; strain 16681), provided by Dr. Tom Monath. The animals were bled each day following virus inoculation and monitored for liver enzyme changes, fever and morbidity. In addition, the presence of infections virus in blood was measured by plaque titration on mosquito cells.

As shown in Table 31 all three virus infected monkeys were found to have significant viremia one to two days following infection. Note however, that from days 3 through 7 all three animals had persistent viremia although no obvious peak was observed. It is unlikely that this represents residual virus since non cell associated viruses generally do not persist in the circulation for

prolonged periods. Furthermore, all of the infected monkeys developed anti-dengue antibodies (titer >160) by 60 days post infection, as reported by the serology division at USAMRIID.

No statistically significant differences in temperature was observed at any time following virus inoculation in any of the infected monkeys (Table 31). Slides were also prepared for differential leukocyte counts; these data will be reported at a future date.

Table 32 summarizes the liver enzyme data. The most obvious difference noted was that one of the infected monkeys (# 921) had a 4 - 5 fold increase in Gamma GTP on days 1-6 following infection. This same monkey also had elevated total bilirubin on days 1 - 6 and presented with the highest viral titers in blood (see Table 31). While there was a trend toward increasing SGOT and SGPT levels on days 2 - 6 in infected monkey #76, the significance of this finding is unclear. No obvious differences were noted in the LDH or alkaline phosphatase levels.

C.3 YEAR THREE OF CONTRACT

The third year of this contract was devoted to a detailed comparison of the efficacy of selected Poly I:C formulations (e.g. free Poly I:C, Poly I:C complexed with poly-L-lysine and carboxymethylcellulose [Poly I:C-LC], liposome encapsulated Poly I:C and dextran conjugated Poly I:C [Poly I:C-Dex]) in primates, human macrophages and mice. These formulations have been compared for their ability to augment i) reticuloendothelial clearance, ii) serum interferon, iii) NK and macrophage cytotoxicity, iv) antiviral activity in human monocytes, and v) resistance to HSV-1 and Banzi virus infections. We have also concentrated our efforts on elucidating the mechanisms by which Poly I:C confers antiviral activity in murine macrophages *in vitro* and resistance to Banzi virus encephalitis *in vivo*. In addition, we have examined the infectivity of other viruses (Punta Toro, Pichinde, and Banzi) in murine macrophages *in vitro*. We have also continued development of the squirrel monkey model of Dengue 2 virus infection.

C.3.1 Comparative studies with various Poly I:C formulations

C.3.1.1 Reticuloendothelial system function

One day following the intravenous inoculation of 50 μg/kg of either Poly I:C-LC or Poly I:C-Dextran mice were injected with chromium labeled sheep red blood cells as previously described and the percent SRBC remaining in the circulation determined at selected

time intervals. As illustrated in Figure 30, the clearance kinetics of Poly I:C-DEX was superior to that observed with Poly I:C-LC. When examined two days after treatment (Table 33), Poly I:C-LC and Poly I:C-DEX appeared to be equally effective in the clearance assay; however, it is important to note that the toxicity observed at 1.25 and 2.5 mg/kg with Poly I:C-LC was not observed with Poly I:C-DEX.

Two days following the intravenous administration of 500 μg/kg of either free or liposome-encapsulated Poly I:C, mice were examined for their ability to clear radiolabeled SRBC. As illustrated in Figure 31, liposome-encapsulated Poly I:C was superior to free Poly I:C in the augmentation of RES function. Likewise, when examined at several dosage levels, the clearance rates in mice receiving 5 μg/kg of liposome-encapsulated Poly I:C was as good as that in mice receiving 500 μg/kg of free Poly I:C (Table 34).

As illustrated in Table 35, free Poly I:C-LC (50 μg/kg) administered two days prior to assay enhanced RES function whereas 500 μg/kg had no effect. This observation has been previously reported (see previous Quarterly Report). In contrast, when Poly I:C-LC (500 μg/kg) was incorporated into liposomes, stimulation of the RES system was still evident. This observation is consistent with the hypothesis that encapsulation causes reduced but sustained levels of Poly I:C-LC in the circulation. Thus, encapsulation may reduce the dose dependent suppressive effect of free Poly I:C-LC. Studies are now underway to evaluate the ability of liposomes to minimize toxicity by sustaining drug release.

C.3.1.2 Serum interferon levels

C.3.1.2.1 Mice

Mice were inoculated intravenously with either 50 or 500 μg/kg of Poly I:C-LC or Poly I:C-DEX and examined for serum interferon levels at 4, 12, and 24 hours post treatment. As illustrated in Table 36, the interferon response was rapid (i.e. maximal levels at 4 hours), and Poly I:C-DEX was as good as Poly I:C-LC when compared at the 500 μg/kg level. In contrast to the above, AVS 5587 was not able to induce interferon at any of the times examined following the administration of 10 mg/kg.

Mice were examined for serum interferon levels at 4, 12 and 24 hours post intravenous administration of Poly I:C. As illustrated in Table 37, both free and liposomal Poly I:C induced interferon 4 hours after administration. Note, however, that liposomal Poly I:C was more effective than free Poly I:C in inducing a prolonged interferon response (see 12 and 24 hour levels). Similar to the observations in mice, squirrel monkeys receiving intravenous

liposomal Poly I:C had higher serum interferon levels than monkeys receiving free Poly I:C (Table 38);
several repeat experiments have verified this finding (Table 39).

Table 40 illustrates the kinetics of serum interferon following intravenous administration of free or liposome-encapsulated Poly I:C-LC. Note that free Poly I:C-LC (500 μg/kg) resulted peak interferon induction eight hours following treatment and by 24 hours dropped tenfold. In contrast, liposome-encapsulated Poly I:C-LC (500 μg/kg) also peaked at eight hours following administration but in this case there was no significant drop in titer by 24 hours. This observation suggests that liposome delivery may act to sustain Poly I:C-LC release over a 24 hour period.

C.3.1.2.2 Primates

A variety of interferon inducers were administered intravenously to primates in an attempt to compare their ability to induce interferon. As illustrated in Table 41, Poly I:C-LC, Poly I:C-DEX and Poly I:C-LIP were comparable in their ability to induce interferon 24 hours after administration. However, when the kinetics of these responses were examined in more detail, Poly I:C-DEX appeared to induce higher interferon levels quicker than did the other Poly I:C formulations (Table 42).

Table 43 presents data on the kinetics of serum interferon after intravenous administration (320 μg/kg) of free, liposome encapsulated and dextran conjugated Poly I:C. As previously reported peak titers of serum interferon were observed 8 hours after treatment with free Poly I:C-LC. In contrast, liposome encapsulated Poly I:C-LC resulted in a higher interferon titers over a longer period. Likewise, dextran conjugated Poly I:C appeared to be as effective as liposome encapsulated preparations.

C.3.1.3 NK and macrophage cytotoxicity

Splenic NK cytotoxicity was examined in mice two days following the intravenous administration of Poly I:C-LC or Poly I:C-DEX. When examined at a dose of 500 μg/kg and effector to target cell ratios of 100, 50 and 25:1, Poly I:C-DEX was as effective as Poly I:C-LC (Table 44).

No significant difference in NK cytotoxicity augmentation was observed in mice receiving either free or liposomal Poly I:C (50 μg/kg) intravenously two days prior to assay. In contrast, liposomal Poly I:C was more consistent in its ability to augment macrophage cytotoxicity (Tables 45 and 46).

Table 47 summarizes data on the effects of encapsulation of Poly I:C-LC on induction of cytotoxic macrophages. Both drugs were equally effective in activating macrophages to become cytotoxic when administered two days prior to assay. You may recall that free Poly I:C-LC had a very transient effect on activation of macrophage cytotoxicity.

C.3.1.4 Phagocytosis

The effects of liposome encapsulation on phagocytosis by peritoneal macrophages is presented in Figures 32-35. Our initial observations suggest that free Poly I:C-LC (Figures 32 and 33) was more effective than liposome encapsulated drug (Figures 34 and 35) following intravenous administration of drug. This observation was made at both three and four days following drug administration. These results may be explained by the fact that liposome encapsulated drug administered intravenously fails to reach the peritoneum.

C.3.1.3 Enhancement of resistance to HSV-1 and Banzi virus infections

Mice receiving Poly I:C-DEX at a dose of either 10 or 1 μg/kg intravenously on the day of virus infection, were rendered fully resistant to a lethal challenge with Banzi virus (Figure 36). Protection was, however, lost when doses as low as 0.1 μg/kg were employed (Figure 37).

As illustrated in Figure 38, liposomal Poly I:C was slightly more effective than free Poly I:C in enhancing resistance to Banzi virus infection. Moreover, as little as 0.01 μg was still able to prevent death in more than 50% of the infected mice (Figure 39).

Figures 40 and 41 illustrate the protective effects of free and liposome-encapsulated Poly I:C-LC in Banzi virus infections. Note that liposome encapsulated drug (500 μg/kg) administered two days prior to virus infection (Figure 40) was as effective as free drug. As illustrated in Figure 41, drug administration on day 0, resulted in complete protection in both the free and liposome-encapsulated forms. Thus, liposome-encapsulated drug was as effective as non-encapsulated drug when administered prophylactically or on the day of virus challenge.

As illustrated in Figure 42, liposome-encapsulated Poly I:C-LC (500 μg/kg) protected 40% of the mice from lethal challenge with HSV-1, but was not as effective as free drug, which protected 80% of the mice. In addition, there was a significant increase in mean survival time in both groups. This pattern was also observed at the lower drug dosage (50 μg/kg).

C.3.1.5 Modulation of HSV-1 induced cytopathic effects in human monocytes

Human monocytes treated *in vitro* with various amounts of free Poly I:C, Poly I:C-LC or Poly I:C-DEX 24 hours prior to infection with HSV-1 were used to determine the ED_{50} values for each drug formulation. While Poly I:C-LC appeared to be more effective than Poly I:C-DEX in this assay, additional studies are needed to determine the exact Poly I:C-DEX levels which are effective at 24 and 48 hours post virus infection (Table 48).

C.3.2 Mechanism of Poly I:C induced antiviral activity in murine macrophages

Macrophages were treated with different doses of Poly I:C for various times before infection with HSV-1. The cytopathic effects of infection was measured two days later using the neutral red dye uptake assay reported previously. Macrophages treated with as little as 0.1 μg Poly I:C per ml demonstrated significant antiviral activity as evidenced by an increase in the viability index; maximal response was achieved at a dose of 1 μg/ml (Figure 43a). For optimal activity, macrophages had to be treated for at least 24 hour prior to infection (Figure 43b). In subsequent experiments macrophages were treated for 24 hours with 5 μg Poly I:C per ml.

We also determined total virus yields (cell associated and supernatant) in Poly I:C treated and untreated macrophages by plaque titration on Vero cells. Table 49 shows that there was only a slight decrease in the number of virus particles isolated from untreated macrophages from 4 to 36 hours after culture. In contrast, by 12 hours Poly I:C treated macrophages had one log less virus and by 24 hours virus titers were two logs lower than those from untreated macrophages. Taken together these data indicate that Poly I:C can induce antiviral activity against HSV-1 in inflammatory macrophages from CD-1 mice and that the results from the neutral red dye uptake assay correlate well with virus yields from infected macrophages. The neutral red dye uptake assay also correlated well with the degree of CPE observed microscopically.

Poly I:C is able to induce the production of a number of cytokines with antiviral activity; these include IFN, IL-1, and TNF. These same cytokines can be made and secreted by activated macrophages. To determine whether Poly I:C-induced antiviral activity against HSV-1 was due to the production of cytokines, inflammatory macrophages were treated with Poly I:C for 24 hours in the presence of antibodies to various cytokines (IL-1, TNF-α, or IFN-α/β). After Poly I:C treatment the cells were infected with HSV-1 and cultured for an additional 48 hours in the presence of the

antibodies. CPE was determined after 48 hours. Controls included Poly I:C treated macrophages cultured in the absence of antibodies and Poly I:C treated macrophages cultured in the presence of antibodies to a cytokine not expected to be involved in antiviral activity (IL-2). The maximum amount of antibodies used in these experiments was enough to neutralize 20 units of IL-1, 10 units of IL-2, 10 units of TNF-α, and 200 units of IFN-α/β. Figure 44 shows that only anti-interferon-α/β antibodies were able to abrogate the protective effects of Poly I:C, indicating that the antiviral activity of Poly I:C was mediated by the production of IFN.

Additional support for the conclusion that Poly I:C's antiviral activity was a result of its production of IFN came from the observation that only IFN-α, IFN-β or IFN-γ were able to induce an antiviral state when added to macrophage cultures prior to infection with HSV-1 (Figure 45). None of the other cytokines (Il-1, Il-2, Il-3, Il-6, or TNF-α) was effective in protecting macrophages from infection with HSV-1 (Figure 45).

To determine whether Poly I:C-induced antiviral activity was due to the production of α-, β- or γ-IFN, macrophages were treated with Poly I:C in the presence of antibodies specific for IFN-α, IFN-β or IFN-γ using the same protocol as above. The data clearly demonstrate that only anti-IFN-β antibodies abrogated the protective effect of Poly I:C, indicating that the antiviral activity of Poly I:C was due to its production of IFN-β (Figure 46). Further support for this conclusion comes from the observation that only IFN-β could be detected in the supernatant from macrophages treated with Poly I:C. In these experiments, macrophages were treated with Poly I:C for 6 hours, the drug was removed by washing the monolayers and 24 hour culture supernatants were assayed for interferon by a neutral red dye uptake assay using a mouse fibroblast line (L929) and VSV. Supernatant from Poly I:C treated macrophages was able to protect L929 cells from infection with VSV and only antibody to IFN-β was able to abrogate this effect, indicating that the supernatant contained IFN-β (Figure 47). Little, if any, IFN-α was detected in the culture supernatant (Figure 47). Similar results were obtained when culture supernatant from macrophages treated with Poly I:C for 15 hours was used.

To determine the time period in which Poly I:C-induced IFN-β exerts its antiviral activity in inflammatory macrophages, anti-IFN-β antibodies were added to cultures of Poly I:C treated cells before or after infection with HSV-1. Controls included Poly I:C treated cells in the absence of anti-IFN antibodies and Poly I:C-treated cells in the presence of antibodies to IFN-γ, which were not expected to abrogate the effects of the drug (see above). Figure 48 shows that when anti-IFN-β antibodies were present in the first 24 hours before infection, together with Poly I:C, they were able to abrogate the protective effects of the drug. However, when the antibodies were added after Poly I:C treatment and infection, no protection was observed (Figure 48). Anti-IFN-γ antibodies had no

effect at any time (data not shown). Thus, Poly I:C exerts its antiviral effects by inducing IFN-β within the first 24 hours. During this time the cells must respond to IFN-β and become resistant to infection with HSV-1.

The results described above clearly indicate that Poly I:C can directly stimulate inflammatory macrophages to become resistant to the cytopathic effects of HSV-1. This antiviral state is mediated solely by the production of interferon-β, which acts in an autocrine manner to induce resistance to HSV-1 infection in macrophages. This conclusion is supported by three observations: i) antibodies to interferon-β completely abrogated the Poly I:C-induced antiviral state whereas antibodies to other interferons or other cytokines did not, ii) addition of interferon to macrophages mimicked the antiviral state induced by Poly I:C, whereas the addition of other cytokines did not and iii) IFN-β was detected in culture supernatants from Poly I:C stimulated macrophages.

C.3.3 Mechanism of Poly I:C-LC induced resistance to Banzi virus encephalitis

The IFN inducer Poly I:C has been shown to be an effective immunomodulator in affording protection against Banzi virus infections in mice (see previous reports). In order to determine its mode of action, we investigated the role played by cellular and humoral components of the immune system in resistance.

To determine the time course of Poly I:C induced IFN production, mice were inoculated intravenously with 10 μg of Poly I:C and examined for serum IFN levels at 0, 4, 8, 12, and 24 hours post treatment. As can be seen in Figure 49, the serum IFN response to Poly I:C was rapid; detectable levels were obtained four hours after administration of drug and reached a maximum at 12 hours. After 12 hours, serum IFN titers gradually diminished over time, reaching levels comparable to controls 24 hours after drug treatment.

In the experiment shown in Table 50, we attempted to determine the role of the spleen in IFN production induced by Poly I:C. Splenectomized or sham operated mice were treated with Poly I:C then challenged with Banzi virus. Early IFN production (IFN induced within 2 hours of Poly I:C administration) in splenectomized mice was not different when compared to sham operated litter mates. In addition, no virus was observed in spleen, serum, or brain of splenectomized or control mice.

Along with the induction of IFN, Poly I:C also induced the activation of natural killer (NK) cells. To determine the extent of NK cell activation, mice were assayed for splenic NK activity 72 hours after drug administration. As can be seen in Table 51, treatment of mice with Poly I:C enhanced NK cell activity by 4 to

6-fold at all effector to target cell ratios tested. Administration of antibody to the ganglioside asialo GM1, which destroys NK cells, completely abolished the potentiation of *in vitro* splenic NK cell activity by Poly I:C.

After evaluating the extent of NK cell activation, studies were conducted to determine the role of these cells in the antiviral activity induced by Poly I:C. Results shown in Figure 50 demonstrated that mice treated with a single dose of Poly I:C or Poly I:C plus asialo GM1 antibody were fully resistant to challenge with a lethal dose (2 LD_{100}) of Banzi virus. In addition, NK cell depletion by treatment with anti-asialo GM1 had no effect on either late IFN production or Banzi virus titers in spleen, brain, and serum of Poly I:C treated mice five days after infection (Table 52). Thus, although Poly I:C activated NK cells, these cells may not play a direct role in resistance to Banzi virus.

Macrophages, like natural killer cells, are activated by Poly I:C. Agents which selectively impair or inhibit macrophage function have been used to elucidate the role of these cells during viral infection. Carrageenan, a sulfated polygalactose extracted from seaweed, is a potent macrophage-toxic substance that causes leakage of lysosomal enzymes into the cytoplasm and consequent cell death. When given intravenously, this drug has a profound depleting effect on tissue macrophages of the reticuloendothelial system, especially spleen and liver macrophages. In the present study, mice were injected intravenously with carrageenan every two days for 6 days, with the final dose given 5 days before treatment with Poly I:C and virus infection. To show that i.v. carrageenan treatment affects tissue macrophages in the spleen and liver the clearance and organ localization of radiolabeled sheep blood cells was investigated. As seen in Table 53, administration of carrageenan resulted in a significant decrease in the clearance rate of SRBC from the circulation, as measured by an increase in the corrected phagocytic index. Moreover, an alteration in the distribution of radiolabeled SRBCs was seen in carrageenan treated compared to control mice; decreased localization was observed in spleen and liver.

To determine if macrophages were required in the augmentation of resistance to Banzi virus infection by Poly I:C, carrageenan or normal saline was administered to mice by intravenous injection in three doses, with the final dose given 5 days before Poly I:C treatment and infection with 2 LD_{100} of virus. Mice were observed for resistance over a 3-week period. As expected, untreated mice succumbed 8-11 days following infection (Figure 51). In contrast, mice treated with Poly I:C or Poly I:C plus carrageenan were protected against infection. However, mice treated with carrageenan alone died significantly sooner than placebo control mice.

In the same experiment, other groups of mice were treated as above, but were sacrificed at 6 days post infection. Spleen, blood, and brain tissues were harvested for quantitation of virus and IFN.

Results summarized in Table 54 demonstrate that, compared to the placebo control groups, a significant increase in brain tissue virus titers was detected in carrageenan treated mice. In addition, late IFN production, which reflects overall viral replication, was elevated. This data correlates with survival. Although carrageenan enhanced virus replication in placebo control groups, no difference in carrageenan and control groups was seen in Poly I:C treated mice. Again, this data correlates with survival data, and indicates that the augmentation of protection by Poly I:C was not compromised by treatment with carrageenan.

Because carrageenan has many biological properties apart from its suppression of mononuclear phagocytes, we felt this data by itself did not negate the role of macrophages in Poly I:C induced resistance to Banzi infection. Consequently, we performed adoptive transfer studies using untreated adherent splenic or peritoneal exudate cell populations or the same cells activated *in vitro* with Poly I:C. Similar experiments have been performed to investigate the role of macrophages in HSV-1 infections. As shown in Figure 52, adoptive transfer of 2.25×10^6 plastic-adherent splenic cells 4 hours prior to virus challenge had no effect on Banzi virus replication in spleens and brains of recipient mice. In another experiment where we looked at survival, infusion of 2.0×10^7 plastic-adherent peritoneal exudate cells did not enhance survival or mean survival times of Banzi virus infected mice compared to placebo control animals (Figure 53). These results, combined with the carrageenan experiments indicate that macrophages do not play a primary role in the protection seen following Poly I:C treatment.

Published reports showed that immune splenic cells cytotoxic for Banzi virus infected L929 fibroblasts develop 6 days following virus infection of C3H/He mice. When these cells were transferred back into C3H/He mice infected 24 hours previously with Banzi virus, a significant reduction in mortality could be seen. Further evidence for a role of T lymphocytes in resistance to Banzi virus comes from experiments in which selective depletion of T lymphocytes with anti-thymocyte serum converted genetically resistant, C3H/RV, mice infected with Banzi virus to the susceptible phenotype. In light of these studies, we examined the effects of Poly I:C in BALB/c athymic nude (nu/nu) mice infected with Banzi virus. As shown in Figure 54, untreated BALB/c and BALB/c athymic nude mice died 8-11 days after virus challenge. In contrast, both BALB/c and BALB/c athymic nude mice treated with a single dose of Poly I:C on the day of virus challenge were fully protected against Banzi virus infection. The data in Table 55 reflect Banzi virus replication in BALB/c and BALB/c athymic nude mice. It can be seen that there was a direct correlation between morbidity in BALB/c and athymic nude mice and Banzi virus titers in spleen, brain, and serum 5 days after virus challenge. Finally, with regards to late IFN production, no difference in late serum IFN levels was observed between BALB/c and BALB/c athymic nude mice treated with Poly I:C. Thus, the protective effect of Poly I:C was not dependent on the presence of functional T cells.

To determine if IFN plays a direct role in the protection afforded by Poly I:C against lethal Banzi virus infection, antibodies to IFN-α/β were administered *in vivo*. In the experiment shown in Figure 55, mice were treated with Poly I:C followed by intravenous administration of antibody to IFN-α/β (1000 neutralizing units/dose). When antibody was given in two doses at 0 and 4 hours after administration of drug, only partial neutralization of the circulating serum IFN was seen. In contrast, when antibody was administered in three doses, at 0, 4, and 8 hours, almost complete neutralization of the circulating serum IFN was observed.

A parallel experiment to evaluate the role of antibody to IFN-α/β in mice challenged with a lethal dose of Banzi virus was performed. As can be seen in Figure 56a, two doses of anti-IFN antibody only partially reversed the *in vivo* effectiveness of Poly I:C in protecting mice from Banzi virus infection. This data correlates with the data presented in Figure 55, which revealed only partial neutralization of Poly I:C induced early IFN following two injections of anti-IFN antibody. In contrast, three doses of anti-IFN antibody dramatically reversed Poly I:C induced resistance to Banzi virus (Figure 56b). This dose is the same as that which neutralized most of the serum IFN induced by Poly I:C (see Figure 55).

To determine the extent of virus replication in mice treated with Poly I:C and/or IFN-α/β, selected tissue were assayed for virus five days after infection. As shown in Figure 57, Poly I:C administered intravenously in a single dose on the day of virus challenge completely inhibited Banzi virus replication in spleen and brain. However, treatment with anti-IFN antibody completely abolished the protective effect of Poly I:C, and resulted in the replication of Banzi virus in spleen and brain to levels comparable to those observed in control mice.

It has been reported that both IFN-α and -β are induced in Poly I:C treated mice. To determine whether both IFN-α and IFN-β played a direct role in the protection against Banzi virus, antibodies to IFN-α/β or to IFN-β were administered in combination with Poly I:C. Poly I:C was given by intravenous injection 4-6 hours prior to infection with Banzi virus). Antibodies to IFN were administered by intravenous injection at 0, 4, and 8 hours following drug treatment. As shown in Figure 58, anti-IFN-α/β dramatically reversed the *in vivo* effectiveness of Poly I:C in preventing virus replication in infected mice, indicating that the IFN induction pathway is a primary mechanism of antiviral resistance. In contrast, when the same total amount of antibody to IFN-β was used, no abrogation of Poly I:C's effects were observed.

The results described above provide clear evidence that the *in vivo* antiviral activity of Poly I:C against Banzi virus is mediated through the production of IFN. Although macrophages, natural killer cells and T cells, which can be activated by IFN and

Poly I:C, may play a secondary role, these cells do not appear to be essential determinants in the protective effects of Poly I:C. Moreover, IFN-α appears to be the predominant IFN type involved in antiviral resistance *in vivo*. These results are in contrast to the role which IFN-β plays in the induction of macrophage intrinsic antiviral resistance following Poly I:C administration *in vitro*.

C.3.4 Infection of murine macrophages *in vitro* with various viruses

Our initial attempts to infect freshly isolated murine macrophages with Banzi virus or Punta Toro virus were unsuccessful. In subsequent experiments we cultured macrophages for four or ten days before infecting them with these viruses and measured CPE by the neutral red dye uptake assay six days later. As can be seen from the results summarized in Tables 56 and 57, macrophages after culture became progressively susceptible to infection as evidenced by a decreased viability index. On day four after culture macrophages could be infected with Punta Toro, HSV-1 and Influenza viruses although they remained resistant to Banzi virus. However, when the culture period was extended to ten days, even Banzi virus infection resulted in CPE.

C.3.5 Dengue 2 virus infection of squirrel monkeys

We have now established a valid model for Dengue 2 virus infection in squirrel monkeys. You may recall that our initial findings with Dengue 2 (strain 16681, received from Fort Detrick) indicated that squirrel monkeys could be infected with this virus as evidenced by viremia and the induction of anti-Dengue specific antibodies, although no consistent changes in fever or liver functions were observed. These results were equivocal due to problems associated with the purity of the virus stock. We have repeated these studies with a strain of Dengue 2 (16681) provided by Dr. Chu from Fort Collins, who has assured us of the purity of this preparation. Results from this study clearly indicate that all infected squirrel monkeys had fever which began on day 3 and lasted through day 12 although no significant changes in liver enzyme levels (Table 58) or in total or differential white cell counts were detected. All of the infected monkeys presented with viremia when assayed 3-5 days after infection and each of the infected monkeys had a significant elevation in anti-Dengue serum neutralizing antibodies on day 30 post infection (Tables 59 and 60).

D. CONCLUSIONS

1. The *in vivo* virus challenge studies suggest that MDP-BSA and Poly-L-lysine conjugates are more effective than free MDP in enhancing resistance to HSV-1 hepatitis and pneumonitis. Moreover conjugated MDP was more effective in enhancing RES functions than was free drug.

2. Squirrel monkeys have been shown to be useful in the evaluation of immunomodulating agents which induce interferon. This primate model will be convenient for the preclinical evaluation of new drugs.

3. The human macrophage antiviral assay is a valuable tool for *in vitro* evaluation of targeted antivirals and immunostimulants. Moreover, this cell lends itself to infection by a variety of RNA and DNA viruses and allows us to easily examine the biological activity of free and conjugated drugs.

4. Conjugation of the nucleoside analogue, PMEA, to poly-lysine was accomplished without any loss of biological activity as determined by the *in vitro* HSV-1/macrophage assay. Moreover, this drug conjugate was more effective than free drug in therapy of viral pneumonitis.

5. Multiple doses of Riker-3M or CL 246738 did not result in sustained augmentation of serum interferon levels and natural killer cell activity. While augmentation was readily apparent after a single dose, multiple doses of Riker-3M or CL 246738 may have an adverse effect on these functions.

6. Neither single nor multiple doses of Riker-3M or CL 246738 were effective in augmenting macrophage cytotoxicity.

7. While a single dose of Riker-3M significantly enhanced phagocytosis, this effect was not sustained following multiple drug doses.

8. Both Riker-3M and CL 246738 were effective in enhancing resistance to banzivirus encephalitis when administered several days prior to virus infection. However, CL 246738 was more effective than Riker3-M in enhancing resistance.

9. Multiple doses of Poly I:C-LC had little , if any, detrimental effects on resistance to Banzi virus encephalitis.

10. Multiple doses of CL 246738 or Riker-3M had no significant detrimental effects on the clearance rate of radiolabeled erythrocytes from the circulation.

11. Liposome encapsulation of Poly I:C enhances the therapeutic potential of this drug. This enhancement may result from selective macrophage delivery and subsequent stimulation of the reticuloendothelial system.

12. Poly I:C-Dextran appears to be as good an interferon inducer in squirrel monkeys as is Poly I:C-LC.

13. AVS-5587 was not as effective as other drugs (*i.e.* Poly I:C-LC, Riker-3M or CL 246738) in our functional assays. In addition neither liposome encapsulation nor conjugation to mannosylated polymers was able to boost the activity of this drug.

14. Poly I:C-DEX is as effective as Poly I:C-LC and perhaps even less toxic based on RES activation studies.

15. Poly I:C-DEX appears to be faster than Poly I:C-LC in its ability to induce interferon in primates.

16. Poly I:C dextran appeared to be as effective as liposome-encapsulated drug in inducing prolonged serum interferon production. Moreover, both formulations were better than free drug.

17. The antiviral activity of Poly I:C in murine macrophages is mediated solely by the induction of IFN-β.

18. IFN-β acts in an autocrine manner to induce resistance in macrophages.

19. There does not seem to be any role for other interferons or cytokines in antiviral resistance in murine macrophages.

20. Macrophages in culture become susceptible to infection with Banzi and Punta Toro viruses and may therefore provide a useful model for evaluating promising immunomodulators or antivirals.

21. The *in vivo* antiviral activity of Poly I:C against Banzi virus is mediated through the production of IFN.

22. Although macrophages, natural killer cells and T cells, which can be activated by IFN and Poly I:C, may play a secondary role, these cells do not appear to be essential determinants in the protective effects of Poly I:C against Banzi virus.

23. IFN-α appears to be the predominant IFN type involved in resistance to Banzi virus *in vivo*.

24. These results are in contrast to the role which IFN-β plays in the induction of macrophage intrinsic antiviral resistance following Poly I:C administration *in vitro*.

25. Dengue 2 virus infection of squirrel monkeys results in an infection similar to that observed in man with clearly defined markers of disease - fever, viremia and production of neutralizing antibody.

E. RECOMMENDATIONS

Based on the data obtained under this contract period we feel that future studies should focus on the immunomodulatory activity of Poly I:C-LC, Riker-3M and CL 246738 in non human primates (squirrel monkeys). These studies should include encapsulation of drugs in liposomes or conjugation to carriers (Poly-L-lysine or dextran) as a means to potentiate their activity and reduce their toxicity.

The development of *in vitro* macrophage models will provide the means to evaluate immunomodulators and antivirals in a cell which plays a key role in resistance to most viral infections. This model should be a component in a primary screen designed to evaluate the clinical potential of new antivirals and immunomodulators.

The development of a Dengue 2 virus model in squirrel monkeys will provide the means to evaluate drug induced resistance to arboviral infections as well as their impact on the immune system. These studies will provide the preclinical data required for approval of immunomodulating and antiviral drugs in man. A more detailed description of our future plans involving the use of these non-human primates for evaluating immunomodulators and/or antiviral drugs can be found in our pending grant renewal.

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Table 1. Susceptibility of LP-BM5 immunosuppressed mice to infection with HSV-1.

Immune Status[a]	Mortality Following HSV-1[b] Infection (dead/total)
No LP-BM5 infection Not immunosuppressed	0/10
LP-BM5 infected Immunosuppressed	12/12

[a] As determined by Con-A mitogen response; IgM in serum; splenomegaly and lymphadenopathy 60 days post LP-BM5 infection.

[b] HSV-1 (approximately 5 x 10^5 pfu) was administered intranasally in 70 ul and the mice observed daily for 21 days.

Table 2. Macrophage involvement in murine AIDS infection.

Inoculum[a] Source	Disease Manifestations: Spleen Weight[b] (mg ± SD)	Con A Response[b] (SI)	Lymph-adenopathy[c]	Susceptibility[c] HSV-1 Infection (% mortality)
Peritoneal Mϕ from uninfected mice	74 ± 13	6	-	0
Peritoneal Mϕ from LP-BM5 infected mice	2039 ± 760	0	++++	100
Splenic Mϕ from uninfected mice	78 ± 12	4	-	0
Splenic Mϕ from LP-BM5 infected mice	1998 ± 300	0	++++	100

[a] Obtained from mock or LP-BM5 infected mice 60 days post-inoculation.

[b] Assays performed 60 days after LP-BM5 inoculation.

[c] Mice were infected with HSV-1 60 days after LP-BM5 inoculation.

Table 3. Serum interferon levels in squirrel monkeys after oral administration of CL 246738 on day 0 and day 4.

Treatment	IFN Titer on Day					
	Pre-Bleed	1	2	3	5	7
Monkey #1 CL 246738	0	31	140	2	3	0
Monkey#2 CL 246738	0	0	78	1	5	0
Monkey#3 CL 246738	0	67	260	1	6	3

Monkeys were gavaged with CL 246738 (25 mg/kg) on day 0 and on day 4 and serum interferon levels were determined using VSV as the challenge virus in primary human foreskin cells. Cell destruction was evaluated using a neutral red dye uptake assay. In this assay, a NIH international standard (10,000 units by plaque reduction) gave a titer of 4706.

Table 4. Conjugated compounds supplied by Dr. Michel Monsigny, Orleans, France.

MDP-BSA Conjugates

1. (MDP)11 - (BSA) (MMB 350)
2. (MDP)11 - (BSA) - (Mannose - 6 Phosphate)33 (MMB 351)
3. Ribavirin - BSA
4. Ribavirin - (Mannose - 6 Phosphate)

Poly-L-lysine Conjugates

1. (Mannose)35 - Poly-L-lysine (PLL - Mannose)
2. (MDP)10 - (Mannose)40 - GG-PL
3. (MDP)47 - (Mannose)25 - GG-PL (MDP - PLL - Mannose)
4. (Ribavirin)20 - Poly-L-lysine
5. (Ribavirin)20 - (Mannose)15 - PLL
6. (Ribavirin)20 - (Mannose)32 - PLL
7. PLL - (Ribavirin)21 - (GLcA)133
8. PLL - (Ribavirin)21 - (Mannose)35 - (GLcA)100
9. PLL - (MDP)30 - (GLcA)136
10. PLL - (Mannose)39 - (GLcA)122
11. PLL - (MDP)30 - (Mannose)42 - (GLc)83
12. 5' - Succinyl - Ribavirin
13. (GLcA)110 - (PMEAGG)20 - Man 6PGG24 - (ACGG)36 - PLK
14. (GLcA)110 - (PMEAGG)20 - (ManGG)55 - (ACGG)5 - PLK
15. PLL (GLcA)110 - (GGMan)60 - (GG Suc Rib)20
16. PLL (GLcA)110 - (GGMan 6P)60 - (GG Suc AZT)20
17. PLL (GLcA)110 - (GGMan 6P)60 - (GG RibTC)20
18. PLL (GLcA)110 - (GGGMan 6P)15 - (GGG PAZTC)20
19. (GLcA)90 - PLK - (GGSuc Selenazol)20 - (GGman)50
20. (GLcA)90 - PLK - (GGSuc Thiazofuran)20 - (GGman)52
21. (GLcA)100 - (PMEA GG)20 - (ManGG)50 - PLK
22. PLL - GGMan - rhuIFNα-B/D (0.741×10^7 U/mg)

PLL = Poly-L-lysine
GLcA = Gluconyl

Table 5. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of MDP or MDP-BSA conjugates on day -2.

Treatment	% Phagocytic Cells			
	Total			
Channel No.	61-255	61-85	86-171	172-255
Saline Control	43	9	27	6
BSA Control	48	9	26	14
Free MDP	42	7	25	10
MMB 350 MDP-BSA	44	8	28	8
MMB 351 MDP-BSA-Man-6-PO_4	44	7	27	9

Mice were given BSA (6.5 mg/kg), free MDP (0.5 mg/kg), MMB 350 (6.5 mg/kg), or MMB 351 (7.3 mg/kg), intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The total number of phagocytic cells and the percentages in the indicated channels were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence while the fluorescence in channels 61-85, 86-171, and 172-255 represent dimly, intermediate and highly fluorescent cells, respectively.

The dosage of conjugate was adjusted so as to administer a equivalent amount of MDP (0.5 μg/kg).

Table 6. Activation of cytotoxic macrophages by free MDP or MDP-BSA conjugates.

Treatment	Statistics	Effector to target ratio		
		40:1	20:1	10:1
Control	CPM	11,200	18,107	17,695
MDP	CPM	7,160	14,924	16,126
	C.I.	36	18	9
	P-value	N.S.	N.S.	N.S.
BSA	CPM	4,689	14,129	16,986
	C.I.	58	22	4
	P-value	N.S.	N.S.	N.S.
MMB-350	CPM	4,748	11,999	14,192
	C.I.	58	34	19
	P-value	N.S.	N.S.	N.S.
MMB-351	CPM	5,126	10,415	15,406
	C.I.	54	42	13
	P-value	N.S.	N.S.	N.S.
Target cells alone:		5,189 CPM		

Mice were injected ip with 10 ug MDP or its equivalent amount (130 ug) on a carrier (MMB-350 or MMB-351) in 0.2 ml pyrogen-free saline 2 days or 4 days before test. Control mice received the same volume of pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and 4×10^3 P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 7. Activation of cytotoxic macrophages by free MDP or MDP-PLL conjugates.

Treatment	Statistics	Effector to target ratio		
		40:1	20:1	10:1
Control	CPM	241147	250740	259691
MDP	CPM	255634	258579	229263
	C.I.	-3	2	12
	P-value	N.S.	N.S.	N.S.
PLL	CPM	241147	250740	247038
	C.I.	2	5	5
	P-value	N.S.	N.S.	N.S.
MDP-PLL	CPM	268102	263538	255756
	C.I.	8	0.3	1
	P-value	N.S.	N.S.	N.S.
Target cells alone:		127384		

Mice were injected ip with 10 ug MDP or its equivalent amount (40 ug) on a carrier, MDP-PLL-Mannose (MDP-PLL) in 0.2 ml pyrogen-free saline 2 days or 4 days before test. Control mice received the same volume of pyrogen-free saline or the carrier (PLL). Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and 4 x 10^3 P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector function.

Table 8. Suppression of HSV-1 replication in lungs of C_3H/Hen mice treated with PMEA conjugated to mannosylated poly-L-lysine.

Treatment[a]	Virus Titer[b] p.f.u./gm.wt.
Control	2.7×10^6
Free PMEA	
50 mg/kg	1.1×10^6
10 mg/kg	1.4×10^6
Conjugated PMEA	
10 mg/kg	1.9×10^5
1 mg/kg	7.4×10^5

[a] PMEA was administered i.v. on days 0, 2, and 4 after intranasal instillation of virus.

[b] Ten percent lung homogenates were prepared from mice 4 days following infection and virus titers were determined by plaque assay on vero cells.

Table 9. Serum interferon responses in mice receiving multiple doses of CL 246738.

Day	Drug Given on Day(s)	Interferon Titer (International Units)
1	0	11,482
4	0,3	<10
7	0,3,6	117
10	0,3,6,9	14
13	0,3,6,9,12	<10

Mice were given CL 246738 (200 mg/kg), orally, on the days indicated, and bled 24 hours later. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described previously.

Table 10. Serum interferon responses in mice receiving multiple doses of Riker-3M.

Day	Drug Given on Day(s)	Interferon Titer (International Units)
1	0	100
4	0,3	<10
7	0,3,6	<10
10	0,3,6,9	25
13	0,3,6,9,12	<10

Mice were given Riker-3M (10 mg/kg), orally, on the days indicated, and bled 24 hours later. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described previously.

Table 11. Splenic NK cytotoxicity following oral administration of Riker-3M compound as a single dose or in multiple doses.

Treatment	Per cent cytotoxicity at Effector : Target Ratios		
No (days)	100:1	50:1	25:1
Control	7	2	1
1 (-2)	46*	29*	22*
1 (-4)	40*	25*	14*
2 (-2, -4)	41*	28*	16*
3 (-2, -4, -6)	31*	20*	13*
4 (-2, -4, -6, -8)	33*	20*	12*

Mice were given 200 μg of Riker-3M compound (10 mg/kg) orally either as single dose or multiple doses of 200 μg each two days apart. Two days after the last treatment, splenocytes were assayed for NK cytotoxicity. YAC cells were used as targets and 4 mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent toxicity was calculated using the formula:

$$100 \times \frac{\text{cpm release in test - spontaneous release}}{\text{maximum cpm release - spontaneous release}}$$

* $p<0.05$

Table 12. Splenic NK cytotoxicity following oral administration of CL 246738 as a single dose or in multiple doses.

Treatment	Per cent cytotoxicity at Effector : Target Ratios		
No (days)	100:1	50:1	25:1
Control	28	19	12
1 (-2)	85*	81*	72*
2 (-2, -4)	77*	68*	51*
3 (-2, -4, -6)	61*	51*	35*
4 (-2, -4, -6, -8)	56*	44*	33*

Mice were given 4 mg CL 246738 (200 mg/kg) orally either as single dose or multiple doses of 4 mg each two days apart. Two days after the last treatment, splenocytes were assayed for NK cytotoxicity. YAC cells were used as targets and 4 mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent toxicity was calculated using the formula:

$$100 \times \frac{\text{cpm release in test - spontaneous release}}{\text{maximum cpm release - spontaneous release}}$$

*$p<0.05$

Table 13. Lack of activation of cytotoxic macrophages by Riker-3M compound.

Treatment No (Days)	Effector to target ratio					
	40:1		20:1		10:1	
	CPM	(CI)	CPM	(CI)	CPM	(CI)
Control	62735		83237		95888	
Riker-3M Compound:						
1 (-4)	66305	(-6)	71497	(14)	83884	(12)
1 (-6)	67619	(-8)	79685	(4)	92294	(4)
1 (-8)	63299	(-1)	73515	(12)	92992	(3)
2 (-4, -6)	67555	(-8)	85534	(-3)	92506	(3)
3 (-4, -6, -8)	66340	(-6)	77725	(7)	86691	(4)

Mice were given 200 ug Riker-3M compound (10 mg/kg) orally either as a single dose or as multiple doses of 200 ug each, two days apart. Macrophage cytotoxicity assay was performed four days after the last treatment. Control mice received the same volume of pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and 4×10^3 P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures} - \text{CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 14. Lack of activation of cytotoxic macrophages by CL 246738.

Treatment No (Days)	Effector to target ratio					
	40:1		20:1		10:1	
	CPM	(CI)	CPM	(CI)	CPM	(CI)
Control	60055		67064		78434	
CL 246738:						
1 (-4)	55972	(7)	71702	(-7)	81878	(-4)
1 (-6)	44661	(26)	58155	(13)	73849	(6)
1 (-8)	48813	(19)	59116	(12)	76994	(2)
2 (-4, -6)	62950	(-5)	64765	(3)	65913	(16)
3 (-4, -6, -8)	58709	(2)	68166	(-2)	74789	(5)

Mice were given 4 mg CL 246738 (200 mg/kg) orally either as a single dose or as multiple doses 4 mg each, two days apart. Macrophage cytotoxicity assay was performed four days after the last treatment. Control mice received the same volume of pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and 4×10^3 P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 15. Clearance and tissue localization of SRBC following treatment with a single or multiple doses of CL 246738

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		K Value	alpha Value	T/2 (min)	Spleen	Liver	Lung
Control	Mean	0.0546	5.56	5.85	177	65	59
	S.D.	0.0172	0.71	1.69	53	10	29
CL 246738 Day -2	Mean	0.0962	6.69	3.20	142	68	21
	S.D.	0.0180	0.44	0.60	31	5	11
	P-Value	<0.01	<0.02	<0.01	NS	NS	NS
CL 246738 Day -2,4	Mean	0.0859	6.02	3.82	106	66	32
	S.D.	0.0343	0.79	1.26	16	21	20
	P-Value	NS	NS	NS	<0.01	NS	NS
CL 246738 Day -2,4,6	Mean	0.0755	6.48	4.14	115	83	41
	S.D.	0.0182	0.67	1.00	20	11	24
	P-Value	NS	NS	NS	<0.05	<0.05	NS
CL 246738 Day -2,4, 6,8	Mean	0.0911	6.74	3.42	93	72	26
	S.D.	0.0216	0.48	0.76	17	8	12
	P-Value	<0.02	<0.02	<0.02	<0.01	NS	<0.05

Mice were given, orally, a single or multiple doses (200 mg/kg) of CL-246 738 two days apart and tested two days after the last dose for clearance of sheep erythrocytes (SRBC) from circulation and their localization in the various organs. Control mice received the same volume of pyrogen free saline. The K value represents the rate of clearance and $t_{1/2}$ represents the time to clear half of the material from the circulation. All groups were compared with the saline-treated group by the Student's T-test.

Table 16. Clearance and tissue localization of SRBC following treatment with a single or multiple doses of Riker-3M.

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		K Value	alpha Value	T/2 (min)	Spleen	Liver	Lung
Control	Mean	0.0751	7.00	4.07	182	61	21
	Std. Dev.	0.0120	0.54	0.58	61	7	7
Lactic Acid Day -2	Mean	0.0927	7.73	3.26	121	72	15
	Std. Dev.	0.0073	0.37	0.26	65	8	4
	P-Value	<0.025	<0.05	<0.025	NS	NS	NS
Riker-3M Day -2	Mean	0.1044	7.67	2.91	82	74	9
	Std. Dev.	0.0118	0.28	0.33	64	3	5
	P-Value	<0.005	<0.05	<0.005	<0.05	<0.005	<0.02
Riker-3M Day -2,4	Mean	0.0889	7.43	3.39	117	68	17
	Std. Dev.	0.0053	0.76	0.21	37	13	13
	P-Value	<0.05	NS	<0.05	NS	NS	NS
Riker-3M Day -2,4,6	Mean	0.0576	6.59	5.26	196	63	43
	Std. Dev.	0.0058	0.23	0.53	31	10	20
	P-Value	<0.02	NS	<0.01	NS	NS	<0.05
Riker-3M Day -2,4 6,8	Mean	0.0834	7.23	3.67	89	75	24
	Std. Dev.	0.0141	0.67	0.65	47	13	10
	P-Value	NS	NS	NS	<0.05	NS	NS

Mice were given Riker-3M (10 mg/kg), orally, on the days indicated, and tested two days after the last dose for clearance of erythrocytes from circulation and their organ localization. Controls received saline or the drug vehicle, 1% Lactic acid. The K value represents the rate of clearance and $t_{1/2}$ represents the time to clear half of the material from the circulation. Groups were compared with the saline control by the Student's T-test.

Table 17. Virological and physiological changes in squirrel monkeys infected with Dengue 1 virus

Treatment	Fever	Liver Enzyme	Viremia (pfu/ml) Day Post-Infection			
			2	3	4	5
Saline Control						
#1	NC	NC	0	0	0	0
#2	NC	NC	0	C	0	0
Virus Infected						
#1	NC	NC	100	<50	125	300
#2	NC	NC	75	50	50	175
#3	NC	NC	<50	<50	75	50

Dengue seronegative squirrel monkeys were prebled and inoculated subcutaneously with 100,000 pfu of the West Pac strain of Dengue 1 virus (courtesy Dr. Ken Eccles, WRAIR). Serum samples were drawn at the days indicated and analyzed for liver enzyme changes or infectious virus in mosquito cells according to the procedure of Mr. Sherm Hasty (USAMRIID).

Table 18. Encapsulation efficiency and stability of liposomal Poly I:C and AVS-5587.

Liposome-encapsulated Drug (concentration)	Hours Post Encapsulation		
	1	24	36
Poly I:C (500 μg/ml)	50% (250 μg/ml)	45% (225 μg/ml)	40% (200 μg/ml)
AVS-5587 (5 mg/ml)	30% (1.5 mg/ml)	24% (1.2 mg/ml)	20% (1.0 mg/ml)

Liposome-encapsulated drugs were prepared by adding drugs (eg. Poly I:C @ 500 μg/ml, or AVS-5587 @ 5 mg/ml) to freeze-dried synthetic lipids prepared by CIBA-GEIGY. The suspension medium consisted of Dulbecco's PBS Poly I:C and 1% sodium bicarbonate for AVS-5587. Encapsulation efficiency was determined spectrophotometrically.

Table 19. Serum interferon responses in mice receiving liposome-encapsulated or free Poly I:C, eight hours post inoculation.

Inoculum	Drug Dose (μg/kg)	Interferon Titer (international units)
Sham Liposomes	--	<10
Free Poly I:C	500	437
Poly I:C/Liposomes	500	589
Poly I:C/Liposomes	50	182

Six week old mice were inoculated i.v. with free or liposomal drug and bled 8 hours later. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described in previous quarterly reports.

Table 20. Serum interferon levels in mice receiving free or liposomal Poly I:C at selected times following drug administration.

Inoculum	Interferon Titer (IU) Hrs Post-Administration		
	4	12	24
Saline	14	<10	40
Sham Liposomes	<10	<10	53
Free Poly I:C	178	74	71
Poly I:C/Liposomes	110	309	229

Six week old mice were inoculated i.v. with free or liposomal drug (500 μg/kg) and bled at the times indicated. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described in previous reports.

Table 21. Splenic NK cytotoxicity following treatment with free or liposome-encapsulated Poly I:C.

Treatment	Per cent cytotoxicity		
	Day 1	Day 2	Day 3
Saline Control	16	-	-
Free Poly I:C	50	68	56
Sham Liposomes	19	14	15
Poly I:C/Liposomes	46	68	55

Mice were given Poly I:C (50 μg/kg) intravenously either in free form or encapsulated in liposomes and an NK cytotoxicity assay was performed one,two or three days thereafter. Control mice received saline or sham liposomes. YAC cells were used as targets and 3 mice per treatment group were analyzed. The assays were done at a 50:1 effector:target cell ratio and are based on the release of chromium from labeled cells. Percent toxicity was calculated using the formula:

$$100 \times \frac{\text{cpm release in test - spontaneous release}}{\text{maximum cpm release - spontaneous release}}$$

Table 22. Clearance of radiolabeled SRBC from mice treated with free or liposome-encapsulated Poly I:C.

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		K Value	alpha Value	T/2 (min)	Spleen	Liver	Lung
Control	Mean	.0920	6.55	3.43	137.65	74.02	16.73
	SD	.0204	1.22	.79	40.77	11.22	9.30
Sham Liposome	Mean	.1055	6.98	3.01	157.81	80.50	11.10
i.v.	SD	.0254	.68	.72	77.73	11.77	6.35
Day - 2	p	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Free Poly I:C	Mean	.1296	7.25	2.42	113.81	77.59	6.14
50 μg/kg i.v	SD	.0283	0.72	0.58	21.05	9.53	4.75
Day -2	p	<0.01	N.S.	<0.05	N.S.	N.S.	<0.05
Free Poly I:C	Mean	.1110	6.97	2.88	101.44	76.92	12.84
5 μg/kg i.v.	SD	.0314	0.45	0.70	43.95	5.89	9.04
Day -2	p	N.S.	N.S.	N.S.	<0.05	N.S.	N.S.
Free Poly I:C	Mean	.1044	7.25	2.93	144.13	88.68	13.33
0.5 μg/kg i.v.	SD	.0149	.33	.39	38.02	9.01	5.56
Day -2	p	N.S.	N.S.	N.S.	N.S.	<0.01	N.S.
Poly I:C/	Mean	.1581	7.04	1.96	60.97	80.15	4.76
Liposomes	SD	.0287	.66	.35	28.73	7.29	2.11
50 μg/kg i.v.	p	<0.001	N.S.	<0.001	<0.01	N.S.	<0.02
Day - 2							
Poly I:C/	Mean	.1480	7.20	2.07	75.28	77.84	4.17
Liposomes	SD	.0212	.37	.29	34.25	6.63	2.44
5 μg/kg i.v.	p	<0.001	N.S.	<0.001	<0.001	N.S.	<0.001
Day - 2							
Poly I:C/	Mean	.1283	8.04	2.50	114.78	85.59	15.18
Liposomes	SD	.0330	1.28	.71	41.19	24.28	12.99
0.5 μg/kg i.v.	p	<0.01	N.S.	<0.01	N.S.	N.S.	N.S.
Day - 2							

Table 23. Activation of cytotoxic macrophages by free or liposome-encapsulated Poly I:C.

Treatment	Expt-1		Expt-2		Expt-3	
	CPM	(CI)	CPM	(CI)	CPM	(CI)
Saline Control	58150		8863		242854	
Free Poly I:C	54542	(6)	9331	(-5)	198758	(18)
Sham Liposomes	67722	(-16)	1747	(80)	237407	(2)
Poly I:C/Liposomes	54763	(6)	1217	(86)	44022	(82)

Mice were given Poly I:C (50 μg/kg), intraperitoneally, either in free form or encapsulated in liposomes and macrophage cytotoxicity assay was performed two days thereafter. Control mice received the same volume of pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and 4×10^3 P388 leukemia cells added to the plate (effector:target cell ratio of 20:1). Cytotoxicity was assayed 48 hours later by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 24. Splenic B and T cell numbers in mice treated with free or liposome encapsulated Poly I:C.

Treatment Group	Cells/Spleen (x 10-7)	B cells		T cells	
		%	Abs. No. (x 10-7)	%	Abs. No. (x 10-7)
Control	6.73	33	2.20	20	1.30
Free Poly I:C	7.67	41	3.21	25	1.99
Sham Liposomes	5.83	30	1.67	25	1.45
Poly I:C/ Liposomes	7.03	33	2.38	27	1.90

Mice were given free or liposome encapsulated Poly I:C (50 μg/kg), intravenously, two days before assaying for the numbers of splenic B and T cells. The numbers of B and T cells were determined using avidin-conjugated monoclonal anti-sIg or anti-Thy 1 antibodies, respectively, and FITC-labeled avidin. The percentage of B and T cells was determined using a Coulter Epics V flow cytometer and the Immuno data analysis program.

Abs. No. = Absolute numbers of B or T cells

Table 25. Serum interferon levels in squirrel monkeys following administration of various formulations of Poly I:C.

Group	Treatment[1]	Interferon Levels (IU)	
		Expt. 1	Expt. 2
1	Sham Liposomes	<10	<10
2	Free Poly I:C	207	73
3	Poly I:C-LC	95	52
4	Poly I:C/Liposomes	466	506
5	Poly I:C-Dextran	133	238

Five monkeys were injected, intravenously, with the indicated formulations of Poly I:C (400 μg Poly I:C/monkey) and the animals were bled one day after drug administration. Serum interferon levels were determined by the neutral red assay using human foreskin fibroblasts.

[1] Pre-bled samples from two monkeys gave serum interferon levels of <10.

Table 26. Serum interferon levels in mice receiving free or liposome encapsulated AVS-5587 at various times following drug administration.

Treatment	Interferon Titer (IU) Hours Post-Administration		
	4	12	24
Control	<10	10	ND
Sham Liposomes	81	39	<10
Free AVS-5587	28	<10	<10
AVS-5587/Liposomes	25	15	<10

Six week old mice were inoculated, intravenously with free or liposome encapsulated AVS-5587 (10 mg/kg) and bled at the times indicated. Serum interferon levels were determined using mouse L cells and the neutral red assay described in previous quarterly reports.

ND = Not done.

Table 27. Clearance and tissue localization of SRBC following treatment with AVS-5587.

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		$T_{\frac{1}{2}}$ (min)	K Value	alpha Value	Spleen	Liver	Lung
Control	Mean	2.91	0.1078	6.91	129.4	68.1	14.9
	Std. Dev.	0.67	0.031	0.72	24.6	8.2	7.7
AVS-5587 (Free)	Mean	2.51	0.1211	6.78	79.8	74.6	9.3
	Std. Dev.	0.32	0.016	0.42	46.5	6.6	6.2
	P-value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Liposomes (Sham)	Mean	2.08	0.1487	7.24	122.0	79.6	7.5
	Std. Dev.	0.44	0.029	0.58	19.2	5.3	3.0
	P-value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
AVS-5587 Liposomes	Mean	2.01	0.1515	7.33	101.58	84.17	9.37
	Std. Dev.	0.31	0.020	0.41	8.48	7.04	4.15
	P-value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Mice were injected, intraperitoneally, with AVS-5587 (10 mg/kg), either free or encapsulated in liposomes 2 days before test for clearance of sheep erythrocytes (SRBC) from circulation and its localization in the various organs. Control mice were injected with pyrogen-free saline. $T_{\frac{1}{2}}$ represents the time to clear half the injected SRBC from circulation and K-value represents the rate of clearance. Both values were obtained by plotting the concentration of injected material remaining in circulation at 2, 4, 6, and 10 minutes after injection.

Table 28. Cytotoxic activity of peritoneal macrophages from mice treated with AVS-5587.

Treatment	Statistics	Effector to target ratio		
		40:1	20:1	10:1
Control	Mean CPM	217921	230540	251115
AVS-5587 (Free)	Mean CPM	219377	252971	253195
	C.I.	-1	-10	-1
	P-value	N.S.	N.S.	N.S.
Liposome (Sham)	Mean CPM	234880	232014	237791
	C.I.	-8	-1	-5
	P-value	N.S.	N.S.	N.S.
AVS-5587 Liposomes	Mean CPM	241892	254921	252473
	C.I.	-11	-11	-1
	P-value	N.S.	N.S.	N.S.

Mice were injected intraperitoneally with AVS-5587 (50 mg/kg), either free or encapsulated in liposomes 2 days before harvesting peritoneal cells. Control mice were injected with pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and 4 x 10^3 P388 leukemia cells added to the plate. Cytotoxicity was assayed by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytotoxicity and cytostasis.

Table 29. Splenic B and T cell numbers in mice treated with AVS-5578, given orally.

Treatment	Cells/Spleen ($\times 10^{-7}$)	B Cells		T Cells	
		%	Abs. # ($\times 10^{-7}$)	%	Abs. # ($\times 10^{-7}$)
Control	2.23	27	0.63	35	0.77
2% $NaHCO_3$	4.87*	33	1.59*	22	1.03
AVS-5587	2.87	29	0.86	21*	0.67

Mice were given AVS-5578 (10 mg/kg) in 2% $NaHCO_3$, by gavage, one day before assaying for the numbers of splenic B and T cells. The numbers of B and T cells were determined using avidin-conjugated monoclonal anti-sIg or anti-Thy-1 antibodies, respectively, and FITC-labeled avidin. The percentage of B and T cells was determined using a Coulter Epics V flow cytometer and the Immuno data analysis program.

Abs. # = Absolute number of B or T cells.

* $P<0.05$.

Table 30. Splenic NK cytotoxicity following intravenous administration of free or liposome encapsulated AVS-5578 on Day -2.

Treatment	% Cytotoxicity at Effector:Target Ratio		
	100:1 ± SD	50:1 ± SD	25:1 ± SD
Control	32 ± 6.7	24 ± 6.5	17 ± 4.9
2% $NaHCO_3$	32 ± 7.1	24 ± 5.0	17 ± 2.7
AVS-5587	42 ± 7.3	33 ± 1.3	25 ± 1.1
Sham Liposomes	38 ± 4.8	30 ± 1.3	20 ± 1.3
AVS-5587/Liposomes	38 ± 1.5	30 ± 1.8	21 ± 1.7

Mice were given free or liposome encapsulated AVS-5587 (10 mg/kg), intravenously, two days prior to assaying for NK cytotoxicity. The drug vehicle was 2% $NaHCO_3$. YAC cells were used as target cells and three mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent cytotoxicity was calculated using the formula:

$$100 \times \frac{\text{cpm released in test - spontaneous release}}{\text{maximum cpm released - spontaneous release}}$$

SD = Standard deviation

Table 31. Temperature, differential leukocyte counts and viremia in squirrel monkeys infected with dengue 2.

Monkey		Temp.	Viremia
Controls	Day 0		
802C		102.0	<10
814C		103.0	<10
Infected			
76		100.4	<10
921		101.2	<10
925		103.4	<10
Controls	Day 1		
802C		104.3	<10
814C		103.2	<10
Infected			
76		103.8	>5000
921		102.6	>5000
925		103.8	>5000

Table 31 continued on next page.

Table 31 continued.

Monkey		Temp.	Viremia
Controls	Day 2		
802C		102.6	<10
814C		104.0	<10
Infected			
76		103.4	325
921		104.0	>5000
925		102.2	875
Controls	Day 3		
802C		103.0	<10
814C		102.8	<10
Infected			
76		102.8	125
921		103.0	400
925		102.2	350

Table 31 continued on next page.

Table 31 continued.

Monkey		Temp.	Viremia
Controls	Day 4		
802C		104.0	<10
814C		103.4	<10
Infected			
76		103.6	100
921		103.4	525
925		102.6	120
Controls	Day 5		
802C		103.4	<10
814C		102.6	<10
Infected			
76		101.6	175
921		102.0	75
925[1]		103.4	-

Table 31 continued on next page.

Table 31 continued.

Monkey		Temp.	Viremia
Controls	Day 6		
802C		102.0	<10
814C		101.6	<10
Infected			
76		101.4	75
921		102.2	-
925		103.0	-
Controls	Day 7		
802C		101.0	<10
814C		100 0	<10
Infected			
76		102.2	125
921		101.7	175
925		103.6	-

Table 31 continued on next page.

Table 31 continued.

Monkey		Temp.	Viremia
Controls	Day 8		
802C		100.4	<10
814C		101.8	<10
Infected			
76		101.8	-
921		101.4	-
925		102.6	-

Monkeys were infected subcutaneously with dengue 2 (1 x 10^6 .f.u.; strain 16681) and bled each day following infection. Viremia was measured by plaque titration on mosquito cells. Monkeys were monitored daily for fever using a rectal thermometer.

[1] Monkey 925 developed a nasal discharge five days after infection.

Table 32. Liver enzyme profiles in squirrel monkeys infected with dengue 2.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 0						
802C	-	147	189	201	114	-
814C	23	361	187	128	62	0.3
Infected						
76	21	235	383	364	310	0.3
921	82	198	248	250	250	0.4
925	24	120	166	230	132	0.2
Controls Day 1						
802C	21	192	244	297	127	0.6
814C	26	154	313	160	111	0.4
Infected						
76	17	146	337	313	325	0.4
921	425	132	205	254	236	1.4
925	33	85	193	271	135	0.5

Table 32 continued on next page.

Table 32 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 2						
802C	24	145	199	296	131	0.4
814C	26	95	250	165	107	0.5
Infected						
76	27	338	443	508	359	0.7
921	435	177	241	231	230	1.7
925	33	130	219	258	139	0.6
Controls Day 3						
802C	-	54	166	230	122	0.4
814C	24	85	193	148	118	1.0
Infected						
76	36	385	778	563	342	1.1
921	436	142	238	192	234	3.0
925	35	68	188	212	131	0.7

Table 32 continued on next page.

Table 32 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 4						
802C	8	68	140	192	140	1.6
814C	22	120	158	116	112	1.4
Infected						
76	32	258	576	433	292	1.3
921	381	159	192	183	198	2.7
925	39	88	202	252	137	1.1
Controls Day 5						
802C	19	132	152	195	137	1.6
814C	19	168	304	161	127	1.6
Infected						
76	32	494	561	540	305	1.3
921	344	112	226	180	224	2.2
925[1]	35	116	215	233	139	1.2

Table 32 continued on next page.

Table 32 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 6						
802C	17	77	136	183	146	1.5
814C	18	98	243	156	144	1.3
Infected						
76	37	462	536	611	330	1.8
921	365	344	208	173	197	3.4
925	31	100	163	210	141	1.0
Controls Day 7						
802C	-	54	120	156	141	-
814C	18	59	173	142	164	0.7
Infected						
76	29	182	326	396	319	0.9
921	-	72	141	138	180	0.6
925	28	99	133	204	144	0.7

Table 32 continued on next page.

Table 32 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 8						
802C	15	57	141	172	160	1.3
814C	19	148	165	136	191	0.6
Infected						
76	33	221	356	386	418	1.1
921	313	201	178	164	300	4.2
925	28	171	142	242	181	1.5

Monkeys were infected subcutaneously with dengue 2 (1×10^6 .f.u.; strain 16681) and bled each day following infection. Viremia was measured by plaque titration on mosquito cells. Monkeys were monitored daily for fever using a rectal thermometer.

[1] Monkey 925 developed a nasal discharge five days after infection.

Table 33. Reticuloendothelial system function following intravenous treatment with Poly I:C-LC or Poly I:C-Dextran administered on Day-2

Dose of Poly I:C (per kg)	Clearance Rate ($T_{\frac{1}{2}}$) in Mice Treated with	
	Poly I:C-LC	Poly I:C Dex
None	2.89 ± 0.73	2.89 ± 0.73
5 μg	2.37 ± 0.29	2.21 ± 0.24
50 μg	1.59 ± 0.16*	1.67 ± 0.36*
500 μg	3.46 ± 0.41	2.82 ± 0.27
1.25 mg	6.07 ± 0.90*	2.85 ± 0.65
2.5 mg	7.21 ± 1.49*	2.91 ± 0.35
5.0 mg	13.40 ± 6.44*	4.77 ± 0.73*

Mice were given Poly I:C conjugates intravenously, two days prior to assaying for the RES function. The data represent the time taken to clear 50 % of injected sheep erythrocytes from blood circulation (± 1 standard deviation).

* $p<0.02$

Table 34. Reticuloendothelial system function following intravenous treatment with Poly I:C in free or liposome encapsulated form administered on Day-2

Dose of Poly I:C (per kg)	Clearance Rate ($T_{\frac{1}{2}}$) in Mice Treated with	
	Free Poly I:C	Poly I:C-Lip
None	3.43 ± 0.79	3.01 ± 0.72
5 μg	2.93 ± 0.39	2.32 ± 0.94*
50 μg	2.88 ± 0.70	2.17 ± 0.34*
500 μg	2.42 ± 0.58*	1.96 ± 0.35*

Mice were given Poly I:C, either in free form or liposome encapsulated, intravenously, two days prior to assaying for the RES function. The data represent the time taken to clear 50 % of injected sheep erythrocytes from blood circulation (± 1 standard deviation).

* $p<0.05$

Table 35. Reticuloendothelial system function following intravenous treatment with free or liposome-encapsulated Poly I:C-LC administered on Day-2.

Dose of Poly I:C (per kg)	Clearance Rate ($T_{\frac{1}{2}}$) in Mice Treated with	
	Free Poly I:C	Liposome-encapsulated Poly I:C
None	3.57 ± 0.88	3.57 ± 0.88
Sham Liposomes	—	3.22 ± 0.46
50 μg	1.96 ± 0.28*	2.67 ± 0.65
500 μg	3.33 ± 1.28	1.83 ± 0.30*

Mice were given free or liposome encapsulated Poly I:C intravenously, two days prior to assaying for the RES function. The data represent the time taken to clear 50 % of injected sheep erythrocytes from blood circulation (± 1 standard deviation).

* $p<0.005$

Table 36. Kinetics of serum interferon levels in mice receiving Poly I:C-LC, Poly I:C-Dextran or PLL-AVS 5587

Treatment	Interferon Titer (IU) Hours Post-Administration		
	4	12	24
Control	<10	ND	ND
Poly I:C-LC 500 µg/kg	646	112	135
Poly I:C-LC 50 µg/kg	631	240	10
Poly I:C-Dextran 500 µg/kg	759	363	34
Poly I:C-Dextran 50 µg/kg	141	155	32
PLL-AVS 5587 10 mg/kg	<10	<10	<10

Mice were inoculated, intravenously with different amounts of Poly I:C-LC, Poly I:C-Dextran or PLL-AVS 5587 and bled at the times indicated. Serum interferon levels were determined using L cells and a neutral red dye uptake assay.

ND = Not Done

Table 37. Kinetics of serum interferon levels in mice receiving free or liposome-encapsulated Poly I:C

Treatment	Interferon Titer (IU) Hours Post-Administration		
	4	12	24
Control	14	<10	40
Sham Liposomes	<10	<10	53
Poly I:C	178	74	71
Poly I:C/Liposomes	110	309	229

Mice were inoculated, intravenously with free or liposome-encapsulated Poly I:C (500 μg/kg) and bled at the times indicated. Serum interferon levels were determined using L cells and a neutral red dye uptake assay.

Table 38. Serum interferon levels in squirrel monkeys receiving free Poly I:C or various formulations of Poly I:C

Treatment	Interferon Titer (IU) 24 Hours Post-Administration
Prebleed	<10
Free Poly I:C	123
Poly I:C-LC	676
Poly I:C-Dextran	302
Sham Liposomes	<10
Poly I:C/Liposomes	257

Monkeys were inoculated, intravenously with free Poly I:C or various formulations of Poly I:C (500 μg/kg) and bled at 24 hours post inoculation. Serum interferon levels were determined using human foreskin fibroblasts and a neutral red dye uptake assay.

Table 39. Kinetics of serum interferon levels in squirrel monkeys receiving free Poly I:C or various formulations of Poly I:C

Treatment	Interferon Titer (IU) Hours Post-Administration			
	4	8	12	24
Free Poly I:C	<10	<10	83	178
Poly I:C-LC	22	62	98	870
Poly I:C-Dextran	0	50	812	346
Sham Liposomes	<10	<10	<10	<10
Poly I:C/Liposomes	<10	<10	45	524

Monkeys were inoculated, intravenously with free Poly I:C or various formulations of Poly I:C (500 μg/kg) and bled at the times indicated. Serum interferon levels were determined using human foreskin fibroblasts and a neutral red dye uptake assay.

Table 40. Kinetics of serum interferon levels in mice receiving free or liposome-encapsulated Poly I:C-LC.

Treatment	Interferon Titer (IU) Hours Post-Administration		
	4	8	24
Control	<10	ND	ND
Sham Liposomes	<10	<10	32
Free Poly I:C-LC 500 µg/kg	1288	2818	309
Free Poly I:C-LC 50 µg/kg	912	316	346
Liposome-encapsulated Poly I:C-LC 500 µg/kg	661	1479	1318
Liposome-encapsulated Poly I:C-LC 50 µg/kg	70	10	36

Mice were inoculated, intravenously with different amounts of free or liposome-encapsulated Poly I:C-LC, and bled at the times indicated. Serum interferon levels were determined using L cells and a neutral red dye uptake assay.

ND = Not Done

Table 41. Serum interferon levels in squirrel monkeys receiving free Poly I:C or various formulations of Poly I:C

Treatment	Interferon Titer (IU) 24 Hours Post-Administration
Prebleed	<10
Free Poly I:C	123
Poly I:C-LC	676
Poly I:C-Dextran	302
Sham Liposomes	<10
Poly I:C/Liposomes	257

Monkeys were inoculated, intravenously with free Poly I:C or various formulations of Poly I:C (500 μg/kg) and bled at 24 hours post inoculation. Serum interferon levels were determined using human foreskin fibroblasts and a neutral red dye uptake assay.

Table 42. Kinetics of serum interferon levels in squirrel monkeys receiving free Poly I:C or various formulations of Poly I:C

Treatment	Interferon Titer (IU) Hours Post-Administration			
	4	8	12	24
Free Poly I:C	<10	<10	83	178
Poly I:C-LC	22	62	98	870
Poly I:C-Dextran	0	50	812	346
Sham Liposomes	<10	<10	<10	<10
Poly I:C/Liposomes	<10	<10	45	524

Monkeys were inoculated, intravenously with free Poly I:C or various formulations of Poly I:C (500 μg/kg) and bled at the times indicated. Serum interferon levels were determined using human foreskin fibroblasts and a neutral red dye uptake assay.

Table 43. Kinetics of serum interferon levels in monkeys receiving free or liposome-encapsulated Poly I:C-LC or Poly I:C-Dextran.

Treatment	Interferon Titer (IU) Hours Post-Administration			
	4	8	12	24
Sham Liposomes	<10	<10	<10	25
Free Poly I:C-LC 320 μg/kg	63	89	28	240
Liposome-encapsulated Poly I:C-LC 320 μg/kg	186	346	323	204
Poly I:C-Dextran 320 μg/kg	295	309	389	229

Monkeys were inoculated, intravenously with free or liposome-encapsulated Poly I:C-LC or Poly I:C dextran (320 μg/kg), and bled at the times indicated. Serum interferon levels were determined using human foreskin fibroblasts and a neutral red dye uptake assay.

Table 44. Splenic NK cytotoxicity following intravenous administration of Poly I:C-LC or Poly I:C-Dextran administered on Day-2

Treatment	% Cytotoxicity at Effector:Target Ratio		
	100:1 ± SD	50:1 ± SD	25:1 ± SD
Control	30 ± 7.6	13 ± 3.5	3 ± 3.1
Poly I:C-LC 500 µg/kg	64 ± 4.2*	53 ± 2.4*	36 ± 2.3*
Poly I:C-Dextran 500 µg/kg	62 ± 8.5*	50 ± 3.8*	37 ± 2.9*
Poly I:C-Dextran 50 µg/kg	51 ± 7.5*	35 ± 5.0*	21 ± 7.0*

Mice were given Poly I:C conjugates intravenously, two days prior to assaying for NK cytotoxicity. YAC cells were used as target cells and three mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent cytotoxicity was calculated using the formula:

$$100 \times \frac{\text{cpm released in test - spontaneous release}}{\text{maximum cpm released - spontaneous release}}$$

SD = Standard deviation

* $p<0.05$

Table 45. Splenic NK cytotoxicity following intravenous administration of free or liposome encapsulated Poly I:C administered on Day-2

Treatment	% Cytotoxicity at Effector:Target Ratio		
	100:1 ± SD	50:1 ± SD	25:1 ± SD
Control	34 ± 5.9	32 ± 7.9	21 ± 5.1
Poly I:C 500 μg/kg	87 ± 0.5*	76 ± 3.7*	65 ± 4.9*
Poly I:C 50 μg/kg	57 ± 8.7*	54 ± 10.4*	34 ± 8.6
Sham Liposomes	39 ± 7.8	39 ± 10.3	19 ± 9.5
Poly I:C Liposomes 500 μg/kg	63 ± 7.0*	51 ± 2.7	45 ± 7.7*
Poly I:C Liposomes 50 μg/kg	70 ± 1.9*	62 ± 4.1*	58 ± 3.6*

Mice were given free or liposome encapsulated Poly I:C intravenously, two days prior to assaying for NK cytotoxicity. YAC cells were used as target cells and three mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent cytotoxicity was calculated using the formula:

$$100 \times \frac{\text{cpm released in test - spontaneous release}}{\text{maximum cpm released - spontaneous release}}$$

SD = Standard deviation

* $p<0.05$

Table 46. Cytotoxic activity of peritoneal macrophages from mice treated with free or liposome-encapsulated Poly I:C

Treatment	Statistics	Expt. #1	Expt. #2
Control	Mean CPM	8863	242854
Poly I:C (Free)	Mean CPM	1747	198758
	C.I.	80%	18%
	P-value	<0.005	N.S.
Liposome (Sham)	Mean CPM	9331	237407
	C.I.	-5%	2%
	P-value	N.S.	N.S.
Poly I:C Liposomes	Mean CPM	1217	44022
	C.I.	86%	82%
	P-value	<0.02	<0.001

Mice were injected intraperitoneally with free or liposome-encapsulated Poly I:C (50 μg/kg), 2 days before harvesting peritoneal cells. Control mice were injected with pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells were washed away and 4×10^3 P388 leukemia cells were added to the plate. Cytotoxicity was assayed by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytotoxic and cytolytic effector functions.

Table 47. Cytotoxic activity of peritoneal macrophages from mice treated with free or liposome-encapsulated Poly I:C-LC.

Treatment	Effector:Target Cell Ratio 40:1 Mean CPM	40:1 CI	20:1 Mean CPM	20:1 CI
Control	11951	--	18967	--
Free Poly I:C-LC 500 μg/kg	8043	33%*	12157	36%*
Free Poly I:C-LC 50 μg/kg	11271	6%	16200	15%
Sham Liposomes	12823	-7%	17728	7%
Liposome-encapsulated Poly I:C-LC 500 μg/kg	7554	37%*	11128	41%*
Liposome-encapsulated Poly I:C-LC 50 μg/kg	10842	9%	16334	14%

Mice were injected intraperitoneally with free or liposome-encapsulated Poly I:C-LC, 2 days before harvesting peritoneal cells. Control mice were injected with pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells were washed away and 4 x 10^3 P388 leukemia cells were added to the plate. Cytotoxicity was assayed by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytotoxic and cytolytic effector functions.

* $p<0.005$

Table 48. Modulation of HSV-1 induced cytopathic effects in human monocytes by free Poly I:C or various formulations of Poly I:C

Treatment	ED_{50} (μg/ml)	
	24 hours	48 hours
Free Poly I:C	ND	>30
Poly I:C-LC	19	4
Poly I:C-Dextran	>30	>30

Human monocytes were treated *in vitro* with various amounts of free Poly I:C, Poly I:C-LC or Poly I:C-Dextran and infected with HSV-1. Cytopathic effects were determined by a neutral red dye uptake assay. The concentration of drug that resulted in a 50% reduction in CPE was determined by linear regression analysis.

ND = Not Done

Table 49. Virus Production in Control and Poly I:C-Treated Inflammatory Macrophages Infected with HSV-1.

Hours of Treatment	Virus Titer (log_{10})[1]	
	Untreated cells	Poly I:C-treated cells
4	5.3	5.1
12	5.0	3.8
24	5.1	2.7
36	4.9	2.8

[1] Macrophages were treated with Poly I:C for the times indicated and then infected with HSV-1 (1.4×10^6 plaque forming units). Forty-eight hours later the cells were frozen and thawed three times to release intracellular virus and the total virus in the supernatant was determined by plaque assay on Vero cells. Untreated cells were cultured but were not treated with Poly I:C.

Table 50. Interferon induction and Banzi virus titers in splenectomized mice following treatment with Poly I:C.

	IFN Titer (IU/ml)[a]				
	Time After Poly I:C		Banzi Virus Titers (PFU/gm or ml)[b]		
	2 Hrs	5 Days	Spleen	Serum	Brain
Normal	115	0	0	0	0
Splenectomized	128	0	NA	0	0

[a] Poly I:C was given intravenously (0.5 mg/kg) to mice. Mice were bled 2 hours and 6 days later, and the serum interferon titers determined. The data represent the average values of 3 mice/group.

[b] Organs were homogenized, made up as 10% (wt/vol) suspensions, clarified at 600 x g, and titrated. Plasma titers are given in PFU/ml. The data represent the average values of 3 mice/group.

Table 51. Potentiation of natural killer cell activity by Poly I:C.

Treatment	Percent Cytotoxicity[a]		
	25:1	50:1	100:1
Placebo-treated	11.5 ± 1.5	20.3 ± 1.1	18.5 ± 1.2
Poly I:C	67.1 ± 5.3[c]	77.6 ± 0.8[c]	72.5 ± 1.6[c]
Asialo GM1[b]	0.8 ± 0.3[c]	1.1 ± 1.6[c]	1.1 ± 0.8[c]
Poly I:C plus Asialo GM1	1.0 ± 0.3[c]	1.4 ± 0.4[c]	1.2 ± 0.4[c]

[a] Determined by ^{51}Cr-release assays against YAC-1 cells at various effector to target ratios. The values represent means ± standard deviations from 3 individual mice per group.

[b] Animals were treated with asialo GM1 antibody 4 hours before Poly I:C treatment.

[c] Statistically significant ($p<0.05$ to $p<0.001$).

Table 52. Effects of Poly I:C on Banzi virus infection in mice treated with asialo GM1 antibody.

Treatment	IFN Titer (IU/ml)	Banzi Virus Titers[a] (Log_{10} PFU/gm of Tissue)		
		Spleen	Serum	Brain
Saline	209	4.49	2.31	2.02
Poly I:C[b]	0[d]	<2.00[d]	<1.00[d]	<2.00
Asialo GM1[c]	309	3.91	2.14	1.76
Poly I:C plus Asialo GM1	0[d]	<2.00[d]	<1.00[d]	<2.00

[a] Organs were removed 5 days post Banzi virus infection, 10% homogenates were prepared and plaque titrations performed on Vero (African Green Monkey kidney) cells. Values represent the mean of 3 mice.

[b] Single intravenous treatments with Poly I:C or normal saline were administered 4 hours prior to virus inoculation.

[c] Asialo GM1 antibody or saline was administered by intravenous injection 8 hours before virus challenge.

[d] Statistically significant difference ($p<0.050$ to $p<0.001$) between this group and the placebo control.

Table 53. Organ uptake of ^{51}Cr-labeled SRBC in livers, lungs, and spleens of carrageenan-treated mice.

Group	Phagocytic Index (corrected)	Organ	Percent Injected Dose[a] per Organ	Percent Injected Dose[a] per gm Wet Weight Tissue
Saline	6.4 ± 0.7	Liver	55.9 ± 4.3	33.6 ± 3.1
		Spleen	6.1 ± 1.5	67.6 ± 22.5
		Lung	2.0 ± 0.8	11.3 ± 4.6
Carrageenan	4.8 ± 0.4[b]	Liver	37.2 ± 10.2[b]	22.8 ± 5.4[b]
		Spleen	1.3 ± 0.5[b]	4.1 ± 1.6[b]
		Lung	4.7 ± 2.6	24.8 ± 12.8

[a] Measured at 10 minutes after 1 x 10^8 ^{51}Cr-labeled SRBC given i.v. All values are means ± standard errors (S.E.) for groups of 5 C3H/HeN male mice.

[b] Significantly differently from values for untreated controls ($p<0.05$ to $p<0.001$).

Table 54. Effects of Poly I:C on Banzi virus infection in mice treated with carrageenan.

Treatment	IFN Titer (IU/ml)	Banzi Virus Titers[a] (Log_{10} PFU/gm of Tissue)		
		Spleen	Serum	Brain
Saline	20	4.96	<1.00	4.18
Poly I:C[b]	0[d]	<2.00[d]	<1.00	<2.00[d]
Carrageenan[c]	537[d]	4.77	<1.00	38.16[d]
Poly I:C plus Carrageenan	0[d]	<2.00[d]	<1.00	<2.00[d]

[a] Organs were removed 5 days post Banzi virus infection, 10% homogenates were prepared and plaque titrations performed on Vero (African Green Monkey kidney) cells. Values represent the mean of 4 mice.

[b] Single intravenous treatments with Poly I:C or normal saline were administered 4 hours prior to virus inoculation.

[c] Carrageenan (0.5 mg) or saline were administered by intravenous injection every 2 days for 6 days, with final dose given 5 days prior to infection.

[d] Statistically significant difference ($p<0.05$ to $p<0.001$) between this group and the placebo control.

Table 55. Effects of Poly I:C on Banzi virus infection in Balb/c and Balb/c athymic (nu/nu) mice.

Strain	Treatment	IFN Titer (IU/ml)	Banzi Virus Titers[a] (Log_{10} PFU/gm Tissue)	
			Spleen	Serum
Balb/c	Saline	29	5.41	1.89
Balb/c	Poly I:C[b]	0[c]	<2.00[c]	<1.00[c]
Athymic (nu/nu)	Saline	437	5.28	1.27
Athymic (nu/nu)	Poly I:C	0[c]	<2.00[c]	<1.00[c]

[a] Organs were removed 5 days post Banzi virus infection, 10% homogenates were prepared and plaque titrations performed on Vero (African Green Monkey kidney) cells. Values represent the mean of 4 mice.

[b] Single intravenous treatment with Poly I:C or normal saline were administered 4 hours prior to virus inoculation.

[c] Statistically significant difference ($p<0.05$ to $p<0.001$) between this group and the placebo control group.

Table 56. Infection of murine macrophages with various viruses after four days in culture.

	Viability Index (%)			
Dilution*	Banzi	Punta Toro	HSV-1	Influenza
None	103	<10	21	14
1-5	92	<10	25	17
1-25	83	37	82	21
1-125	88	104	71	111

* The multiplicities of infection in the undiluted samples were 25 for Banzi, 1 for Punta Toro, 10 for HSV-1 and 250 (hemagglutination units) for Influenza.

Table 57. Infection of murine macrophages with various viruses after ten days in culture.

	Viability Index (%)			
Dilution*	Banzi	Punta Toro	HSV-1	Influenza
None	<10	<10	<10	<10
1-5	<10	28	<10	<10
1-25	<10	95	<10	100
1-125	<10	73	<10	124

* The multiplicities of infection in the undiluted samples were 25 for Banzi, 1 for Punta Toro, 10 for HSV-1 and 250 (hemagglutination units) for Influenza.

Table 58. Temperature and liver enzyme profiles in squirrel monkeys infected with Dengue 2 (strain 16681).

		Liver Enzymes	
	Temperature	AST	ALT
Uninfected	102.8 ± 0.8	199 ± 93	232 ± 73
Infected			
Day -1 - Day 2	103.0 ± 0.5	187 ± 54	145 ± 53
Day 3 - Day 12	104.2 ± 0.6*	207 ± 70	187 ± 97
Day 13 - Day 14	103.5 ± 0.7	139 ± 36	110 ± 31

Monkeys were infected on day 0 with Dengue 2 (1×10^6 pfu) and their temperature was recorded daily for 14 days. Serum samples were collected daily for 14 days and analyzed for serum transaminase activities (AST or ALT). Pre-infection temperatures and serum samples were also collected on day -1. The results for the uninfected monkeys (n=2) represents the mean values for all samples taken over the 15 day period. The results for the infected monkeys (n=3) represents the mean values for the samples taken on the days indicated.

* $p<0.001$

Table 59. Viremia in squirrel monkeys infected with Dengue 2.

Days Post Infection	Titer (pfu/ml) Monkey Number 1532	1553	1624
1	100	50	150
2	<10	20	150
3	1175	1575	600
4	250	775	425
5	<10	200	100
6	<10	25	<10
7	150	<10	<10
8	<10	<10	<10
9	<10	<10	<10
10	<10	<10	<10

Heparinized blood from infected monkeys was tittered on monolayers of LLC-MK2 cells in 12 well tissue culture plates. One tenth of an ml of plasma was added to each monolayer and adsorbed for 90 minutes. An agarose overlay was added and the infected cells incubated for 5 days, at which time a second agarose layer containing neutral red was added. Plaques were counted at each dilution and the number of pfu/ml calculated.

Table 60. Neutralizing anti-Dengue 2 antibody titers in squirrel monkeys thirty days after infection.

Treatment	Plaque Reduction Neutralization Titer (PRNT) 50%	80%
Uninfected		
Monkey 1537	<10	<10
Monkey 1671	<10	<10
Infected		
Monkey 1532	1280	640
Monkey 1553	640	640
Monkey 1624	1280	640

PRNT titers were determined using LLC-MK2 cells grown in 12 well tissue culture plates. Monolayers were infected with Dengue 2 (100 plaques per monolayer) either in the presence or absence of serum from uninfected or infected monkeys. The dilution which resulted in a 50% or 80% reduction in plaque number was determined. An internal positive control consisting of pooled serum from hyper-immunized monkeys gave a 50% titer of 2560 and a 80% titer of 640.

Figure 1. Lymphadenopathy in LPBM-5 infected mice.

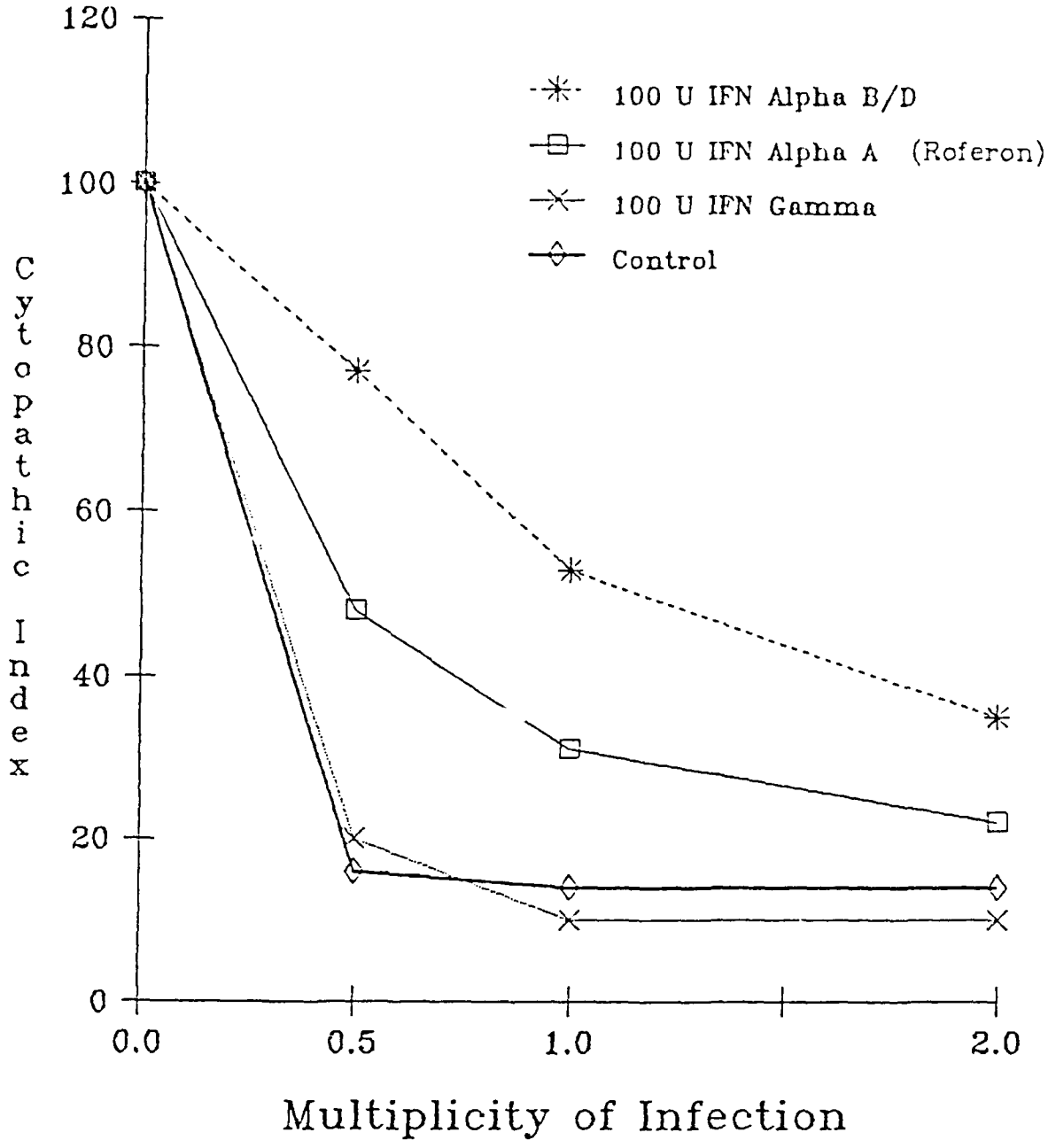
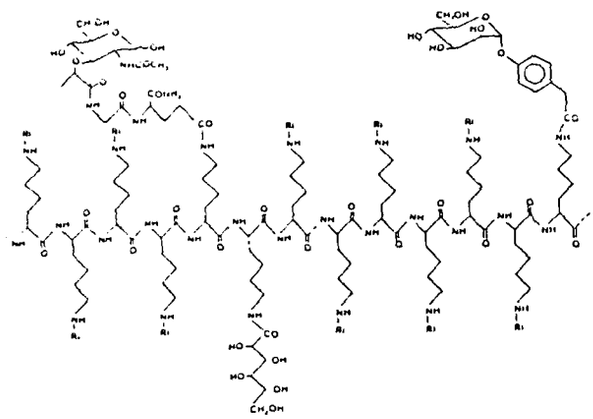


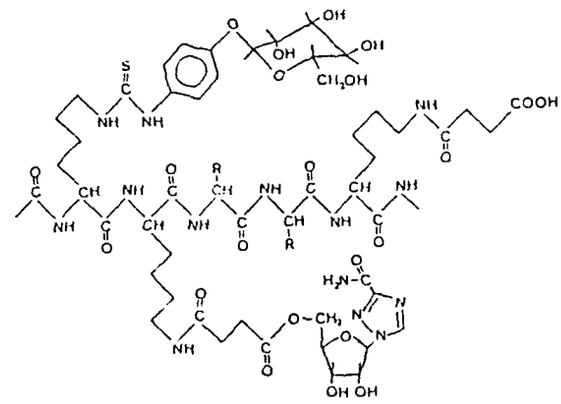
Figure 2. Inhibition of HSV-1 cytopathic effect in differentiating human monocytes.

Monocytes were cultured for 10 days and then added to microtiter plates (1×10^5 cells/well). Cells were incubated overnight with interferon or media for 18 hours. Treated cells were then washed and infected with HSV-1 (VR/3).

MICHEL MONSIGNY
ORLEANS, FRANCE



(MDP)-(MANNOSE)-POLYLYSINE



(SUCCINYL-RIBAVIRIN)-(MANNOSE)-POLYLYSINE

R1=MDP, GlcA OR MANNOSE DERIVATIVE
R=RIBAVIRIN

Figure 3. Chemical structure of gluconoylated and mannosylated Poly-L-Lysine substituted with MDP or ribavirin residues.

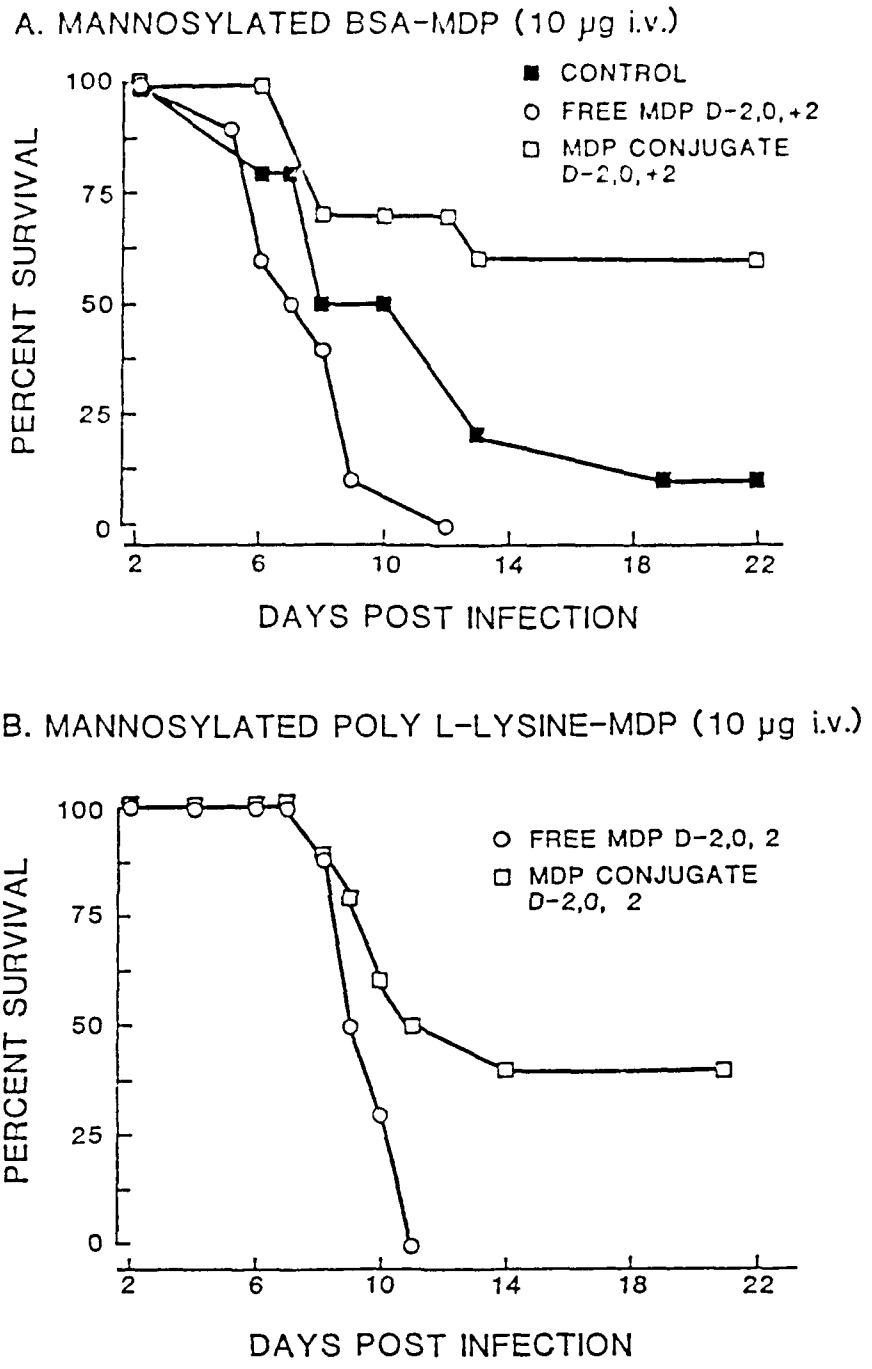


Figure 4. Treatment of HSV-1 induced hepatitis with MDP conjugates.

Mice received 10 ug i.v. of either free or conjugated MDP 2 days prior to, on the day of and 2 days following infection. All mice received 1 LD_{80} of HSV-1 (MB strain) intravenously on day 0 and were followed for 21 days. Ten mice per group were evaluated using Wilcoxon rank analysis.

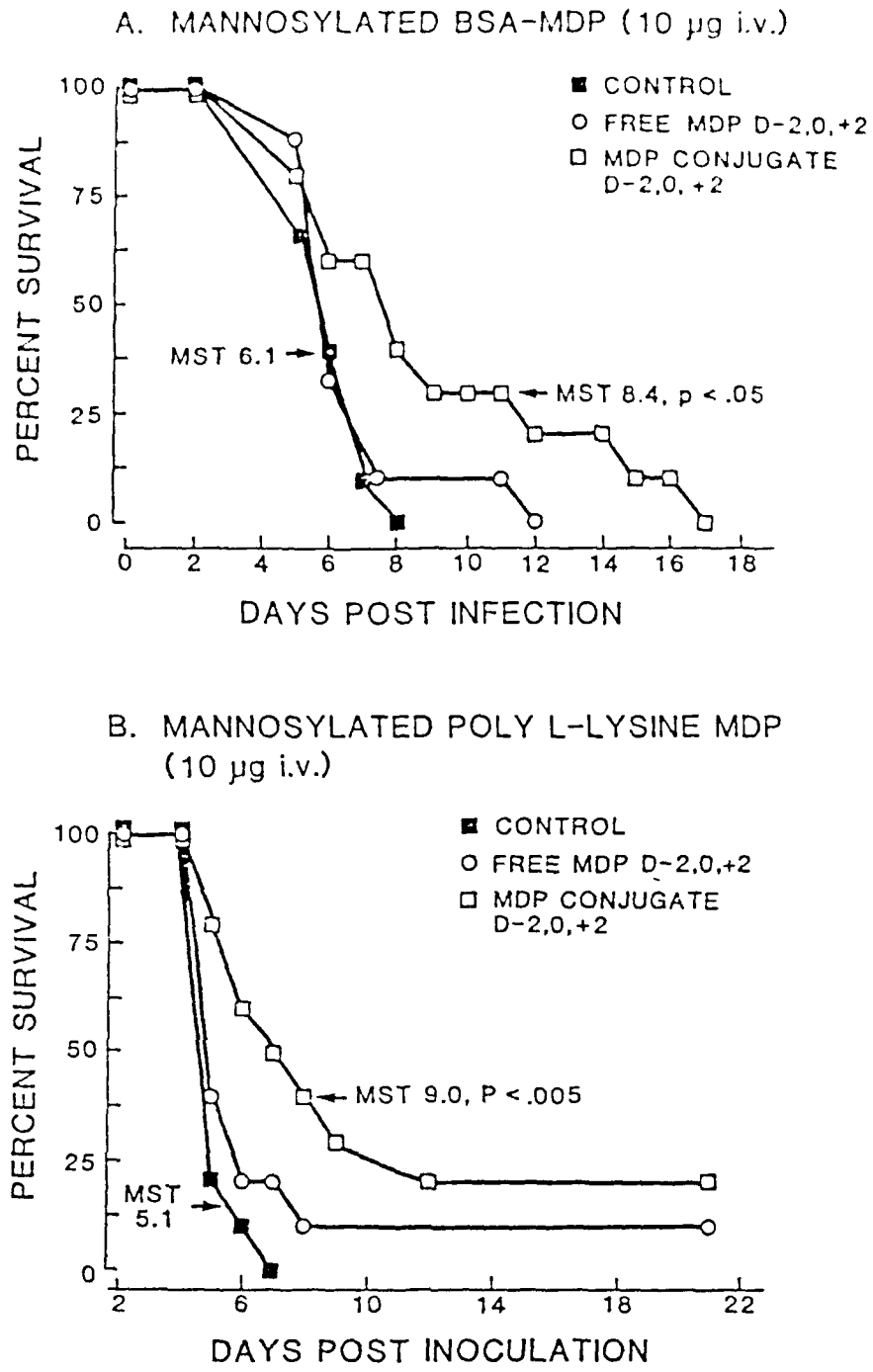


Figure 5. Treatment of HSV-1 induced pneumonitis with MDP conjugates.

Mice received 10 ug i.v. of either free or conjugated MDP 2 days prior to, on the day of and 2 days following infection. All mice received 1 LD_{80} of HSV-1 (VR/3 strain) intranasally on day 0 and were followed for 21 days. Ten mice per group were evaluated using Wilcoxon rank analysis.

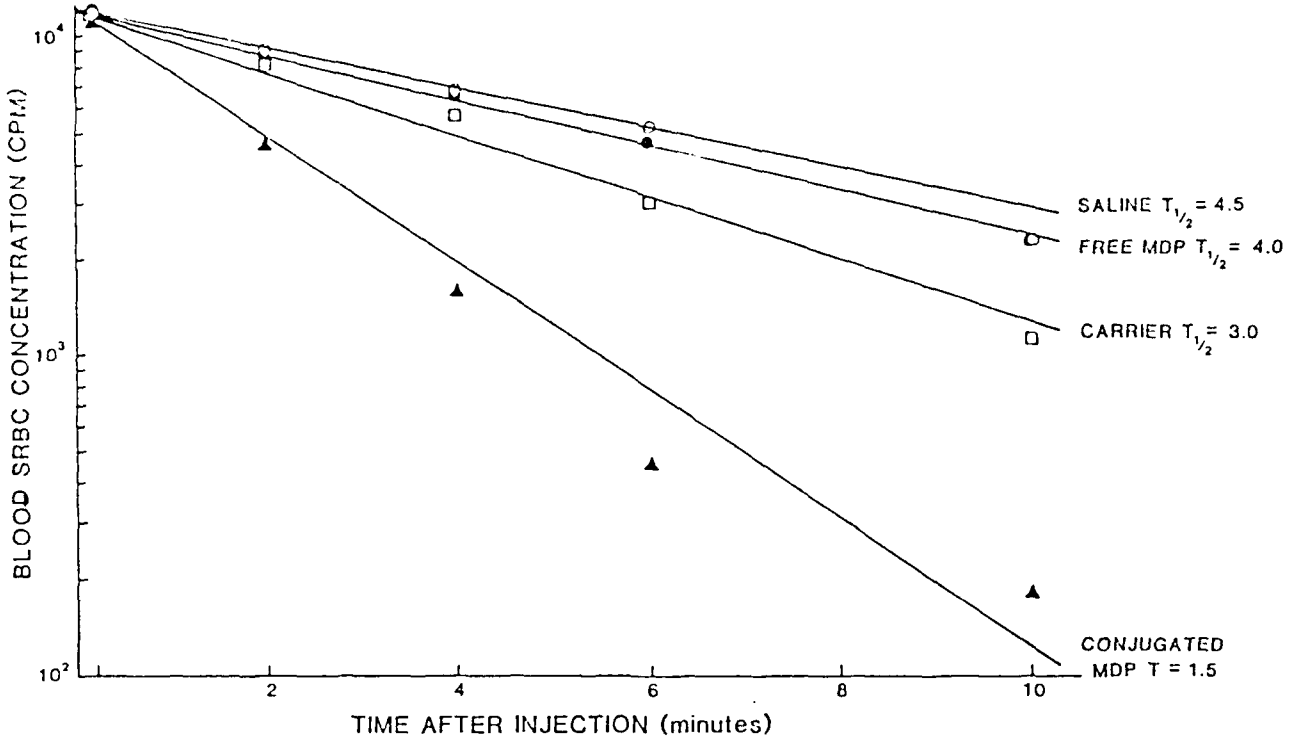
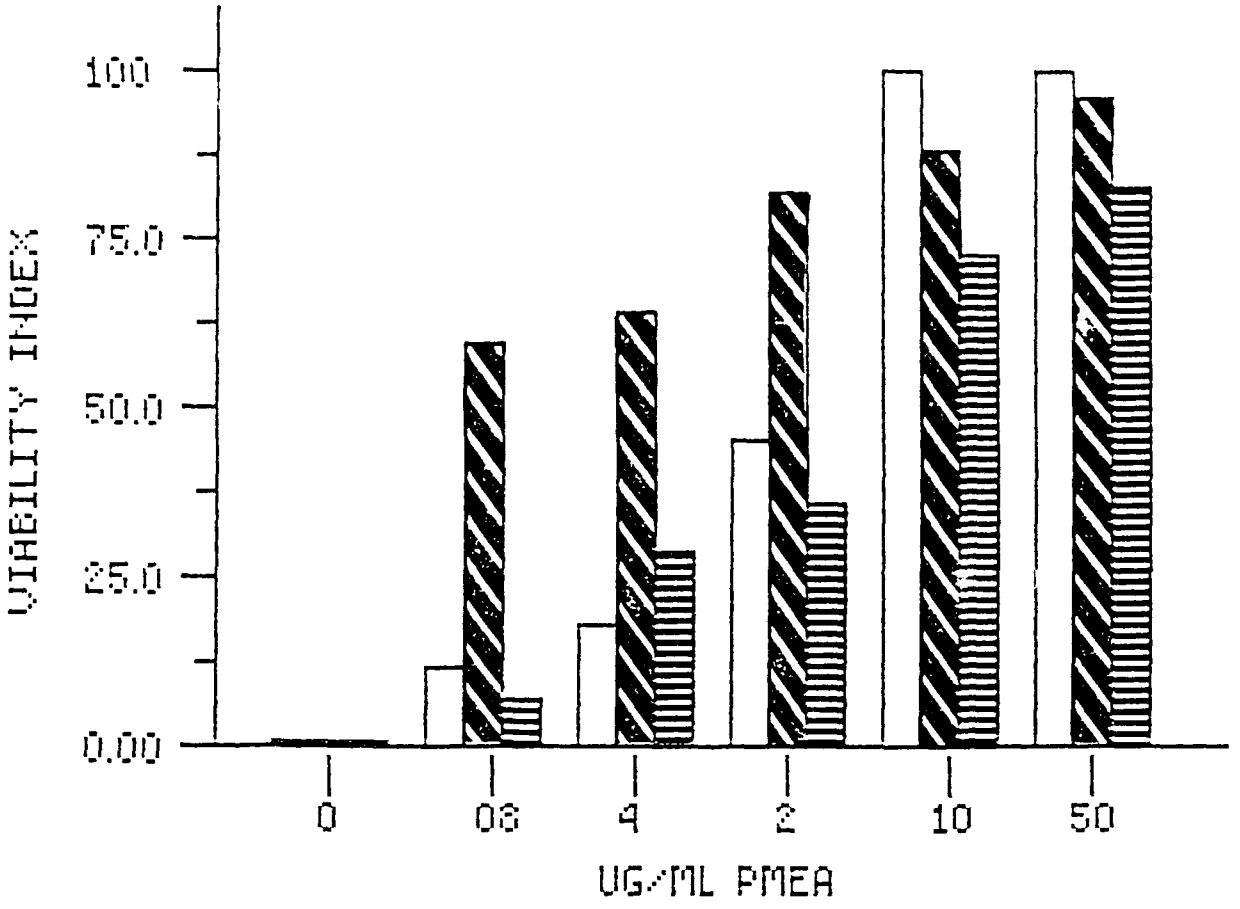


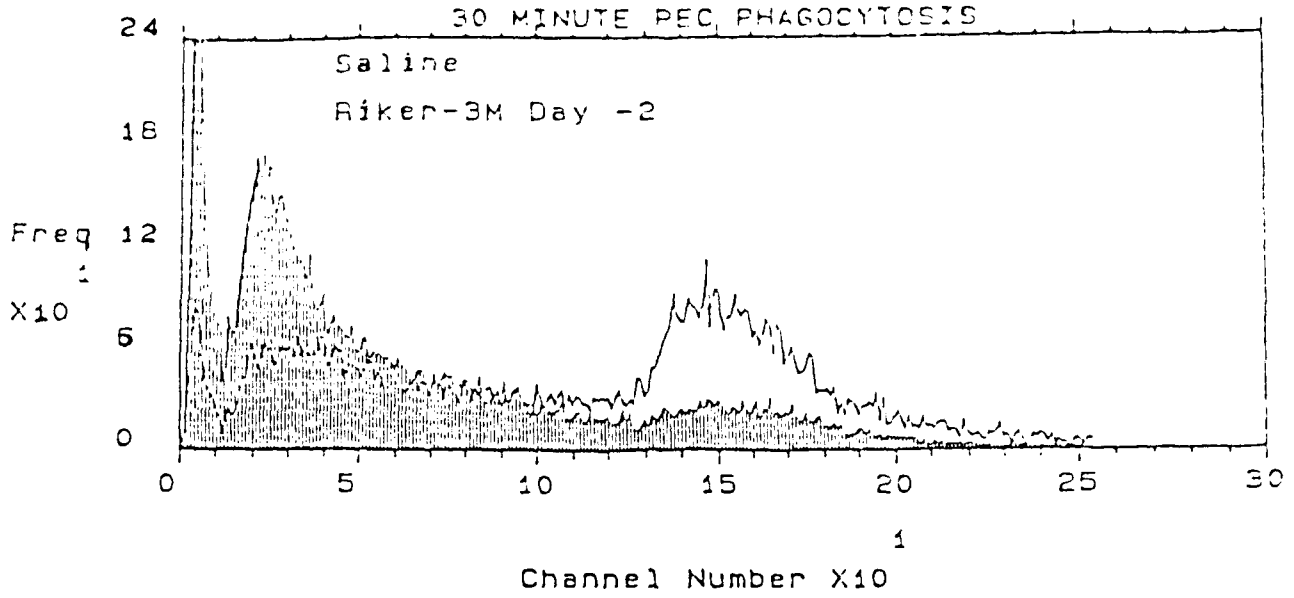
Figure 6. RES stimulation by mannosylated Poly-L-lysine MDP conjugate.



1. Free PMEA ▭.
2. Mannosylated Poly-L-Lysine PMEA ((GLcA)110 - (PMEAGG)20 - (ManGG)55 - PLK) ▨.
3. Mannose (6 PO)4 Poly-L-Lysine PMEA ((GLcA)110 - (PMEAGG)20 - (Man6PGG)24 - PLK) ▤.

Figure 7. Free versus conjugated PMEA in the treatment of human macrophages infected with HSV-1.

Monocytes were cultured for 10 days and then added to microtiter wells (1×10^5 cells/well). Cells were infected with HSV-1 (VR/3) at a multiplicity of infection = 1 and then incubated in the presence of drug for 24 hours. Viability was determined using neutral red dye uptake. The viability index was determined by extracting neutral red from cell monolayers and then quantitating using a colorimetric procedure described in Appendix.

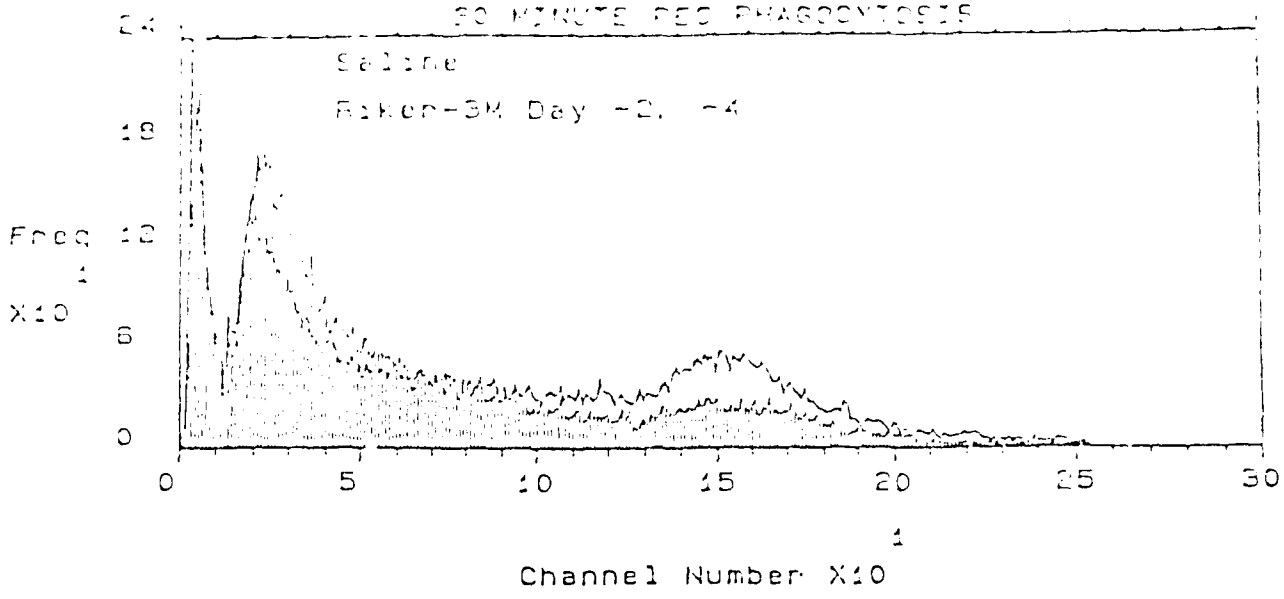


Treatment	Total	Log 1	Log 2	Log 3
	% Phagocytic Cells			
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2)	70*	9	44*	18

Figure 8. Phagocytosis by peritoneal exudate cells following oral administration of Riker-3M on day -2.

Mice were given Riker-3M (10 mg/kg), orally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

* $p < 0.01$

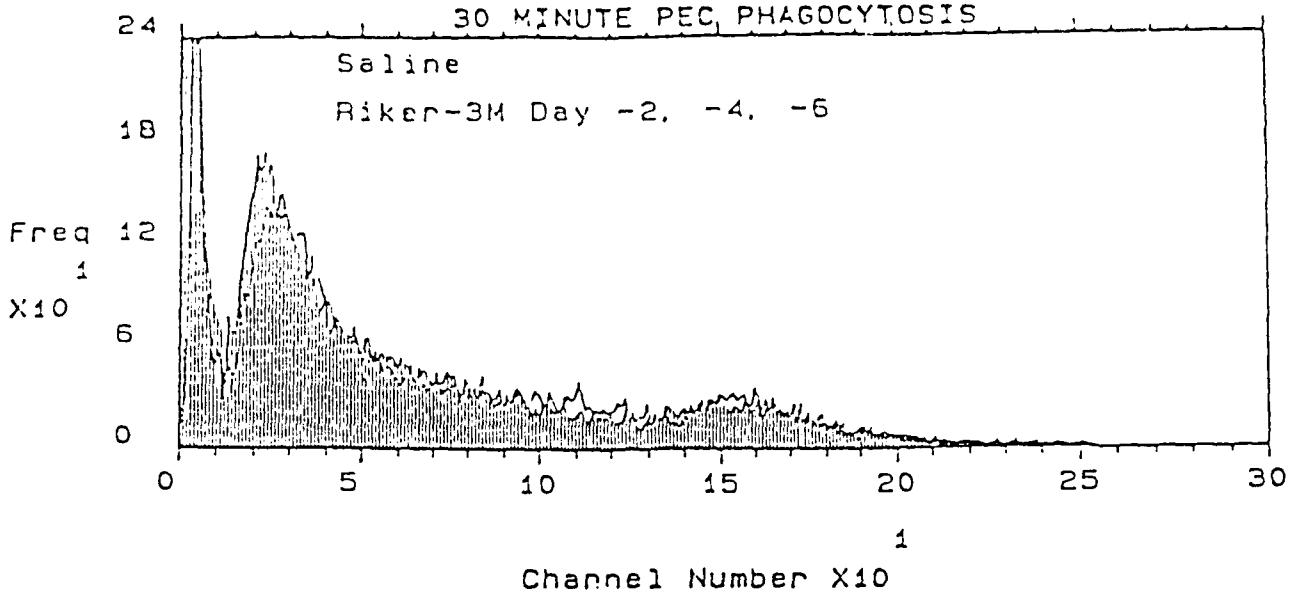


Treatment	Total	Log 1	Log 2	Log 3
	% Phagocytic Cells			
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2,-4)	53	9	33*	11

Figure 9. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M on days -2 and -4.

Mice were given Riker-3M (10 mg/kg), intraperitoneally, two and four days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

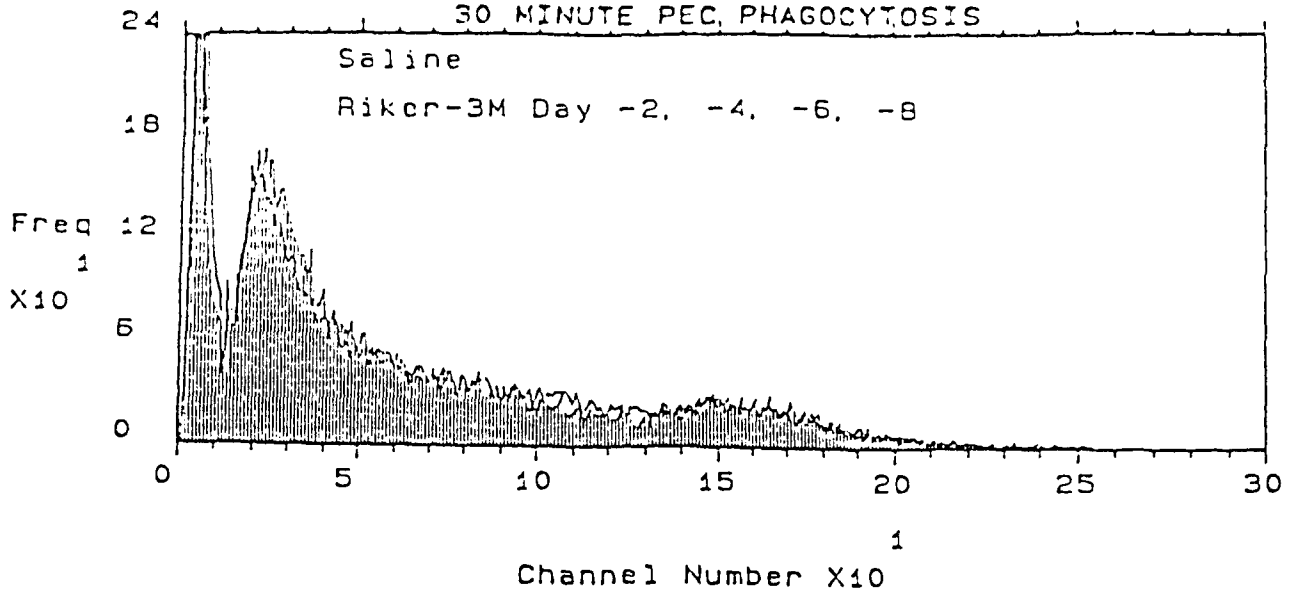
* $p < 0.05$



Treatment	% Phagocytic Cells			
	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2,-4,-6)	37	9	22	6

Figure 10. Phagocytosis by peritoneal exudate cells following intraperitoncal administration of Riker-3M on days -2, -4 and -6.

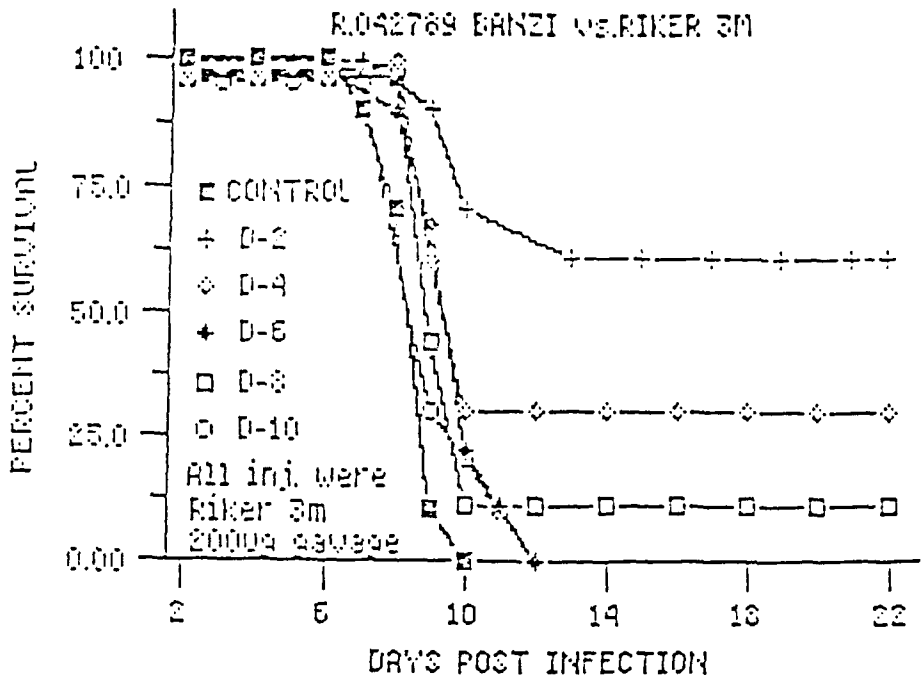
Mice were given Riker-3M (10 mg/kg), intraperitoneally, two, four and six days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



	% Phagocytic Cells			
Treatment	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2,-4,-6,-8)	46	10	25	12

Figure 11. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M on days -2, -4, -6 and -8.

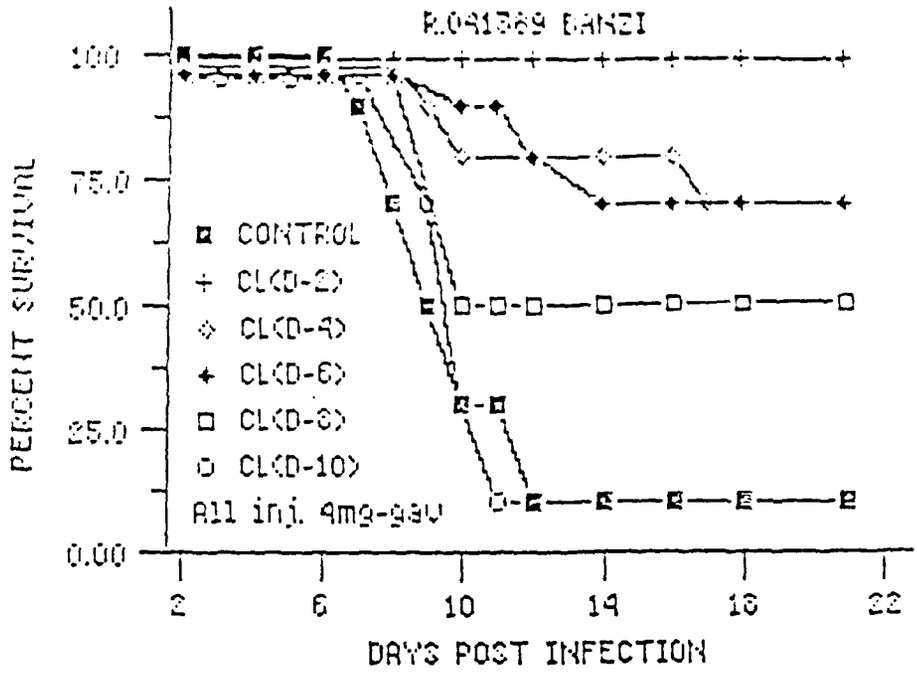
Mice were given Riker-3M (10 mg/kg), intraperitoneally, two, four, six and eight days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



Treatment	Geometric Mean Survival Time (days)	P-value
Control- 1% lactic acid	8.7	—
Riker-3M 10 mg/kg prior to infection		
day-2	15.9	<.005
day-4	12.0	<.05
day-6	9.8	NS
day-8	10.2	NS
day-10	9.6	NS

Figure 12. Prophylactic activity of Riker-3M in Banzi encephalitis.

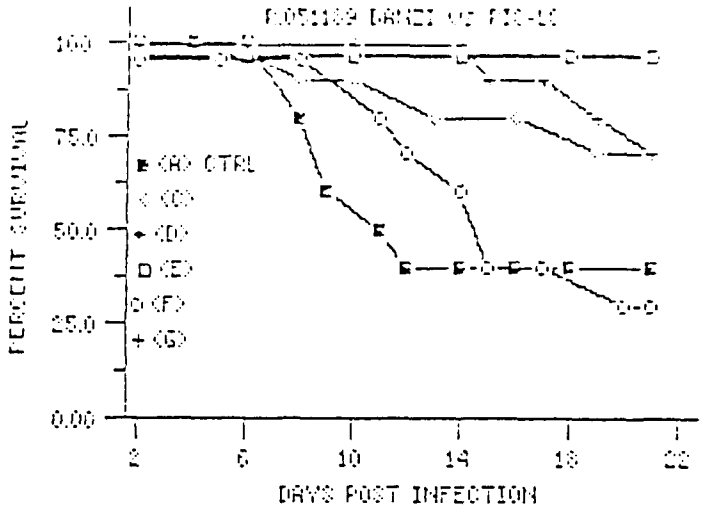
Mice received by gavage 10 mg/kg of Riker-3M in a 1% lactic acid carrier on the day indicated. Mice were challenged intraperitoneally with 1 LD_{80} of virus on day 0 and examined daily for 21 days. Ten mice were included in each group. Significance levels were determined using Wilcoxon rank analysis.



Treatment	Geometric Mean Survival Time (days)	P-value
Saline Control	10.2	—
CL 246738 200 mg/kg prior to infection		
day-2	21.0	<.001
day-4	17.5	<.01
day-6	17.7	<.01
day-8	14.5	<.05
day-10	10.6	NS

Figure 13. Prophylactic activity of CL 246738 in Banzi encephalitis.

Mice received by gavage 200 mg/kg of CL 246738 on the day indicated. Mice were challenged intraperitoneally with 1 LD_{80} of virus on day 0 and examined daily for 21 days. Ten mice were included in each group. Significance levels were determined using Wilcoxon rank analysis.



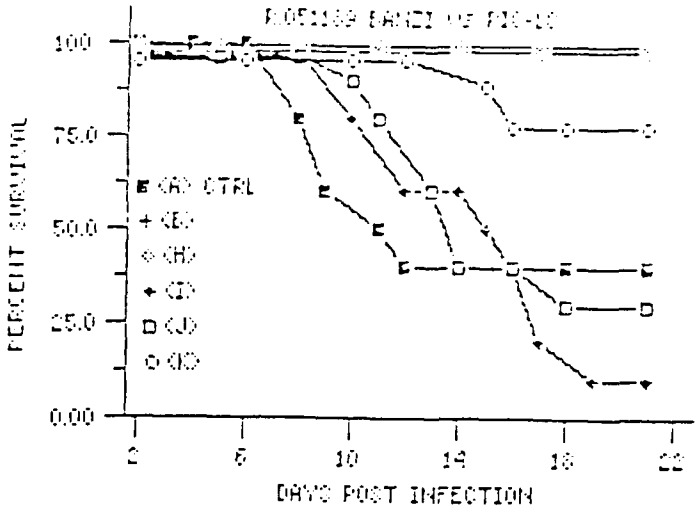


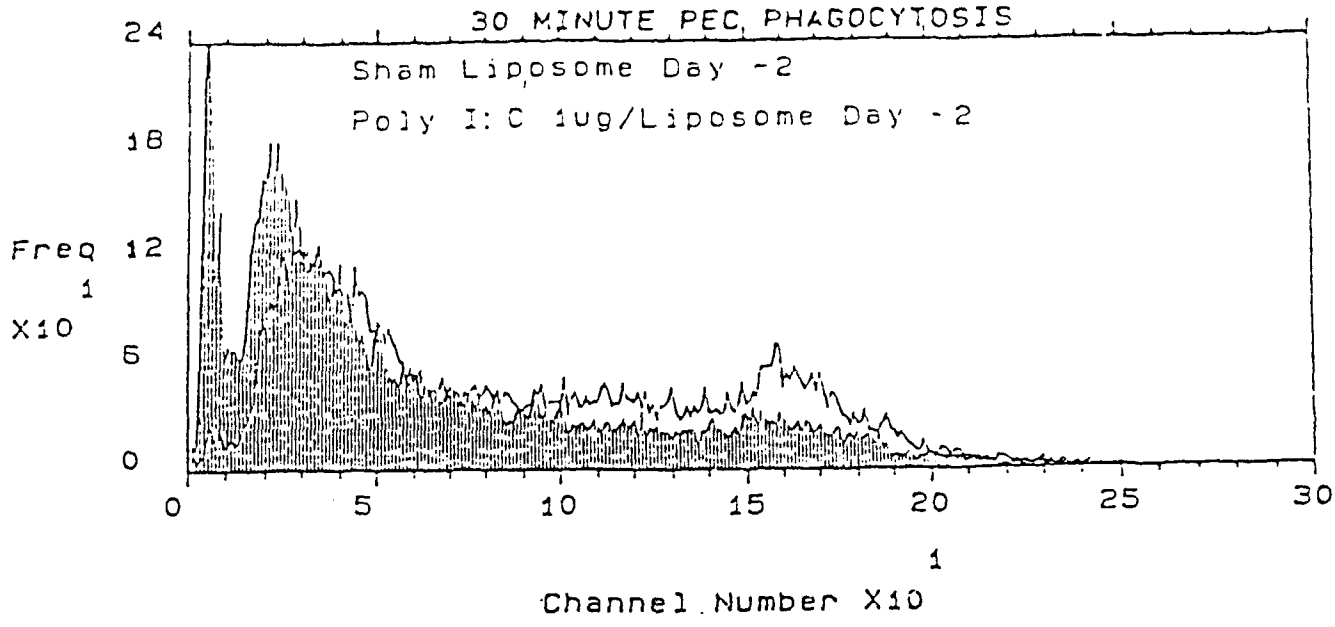
Figure 14a

Group	Day of treatment
A.	-10, -5, 0, +5, +10 (saline control)
C.	-10, -5, 0, +5, +10
D.	-11, -6, -1,+4, +9
E.	-12, -7, -2,+3, +8
F.	-13, -8, -3, +2, +7
G.	-14, -9, -4, +1, +6

Figure 14b

Group	Day of treatment
A.	-20, -15, -10, -5, 0, +5, +10 (saline control)
B.	-20, -15, -10, -5, 0, +5, +10
H.	-21, -16, -11, -6, -1, +4, +9
I.	-22, -17, -12, -7, -2, +3, +8
J.	-23, -18, -13, -8, -3, +2, +7
K.	-24, -19, -14, -9, -4, +1, +6

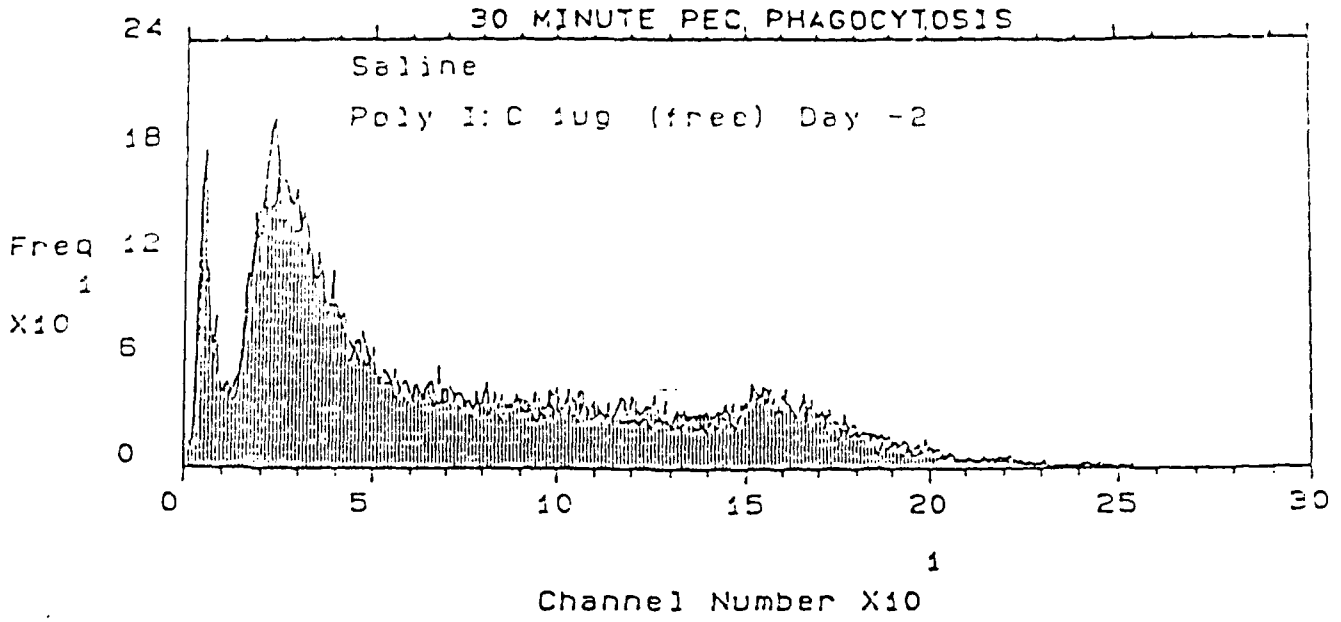
Figure 14. Resistance to Banzi virus following five (Figure 14a) or seven (Figure 14b) i.v. injections with Poly I:C-LC.



Treatment	% Phagocytic Cells			
	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Sham Liposomes	47	10	22	6
Poly I:C/Liposomes	56	11	36	10

Figure 15. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated Poly I:C on day -2.

Mice were given liposome encapsulated Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

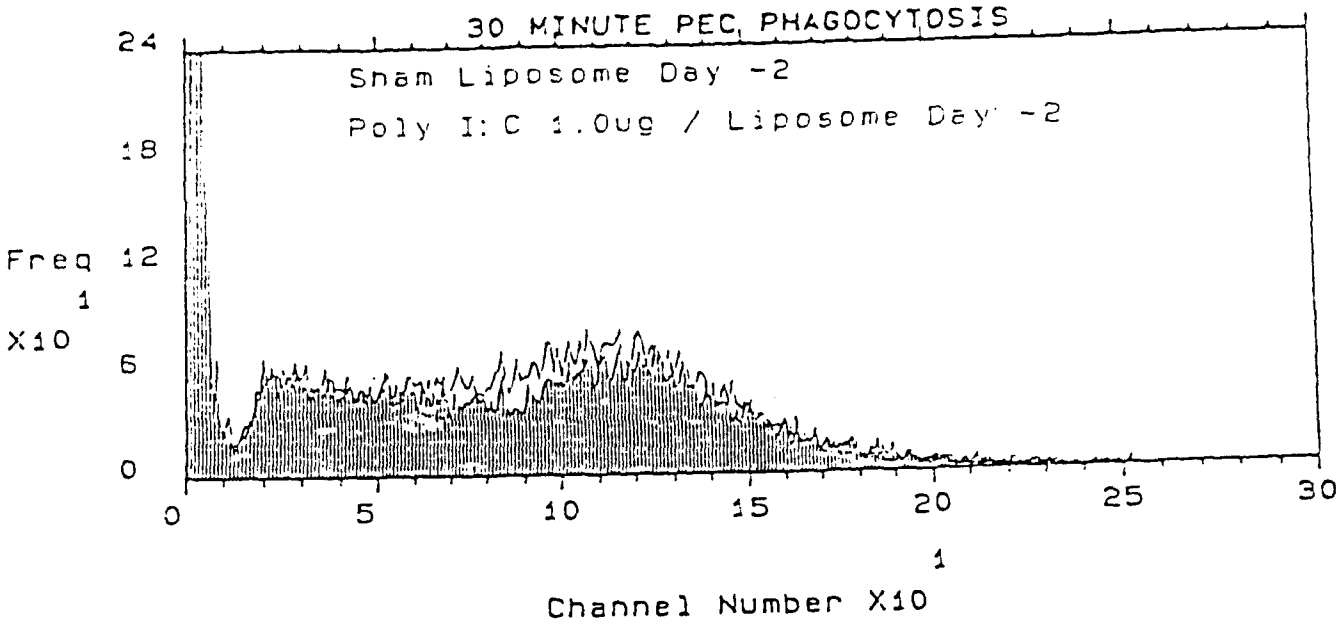


% Phagocytic Cells

Treatment	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Control	47	10	30	7
Free Poly I:C	45	10	27	9

Figure 16. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of free Poly I:C on day-2.

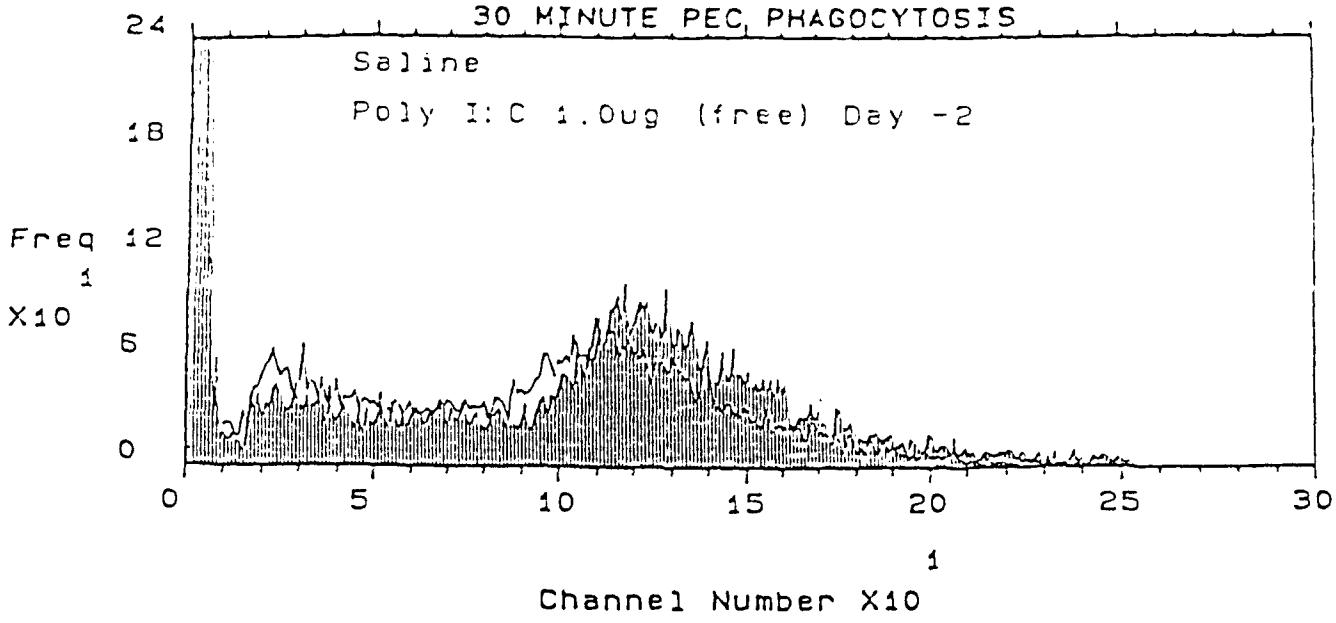
Mice were given free Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



Treatment	% Phagocytic Cells			
	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Sham Liposomes	54	11	38	6
Poly I:C/Liposomes	60	13	44	3

Figure 17. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated Poly I:C on day -2.

Mice were given liposome encapsulated Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



Treatment	% Phagocytic Cells			
	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Control	70	10	51	8
Free Poly I:C	51	8	39	5

Figure 18. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of free Poly I:C on day-2.

Mice were given free Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

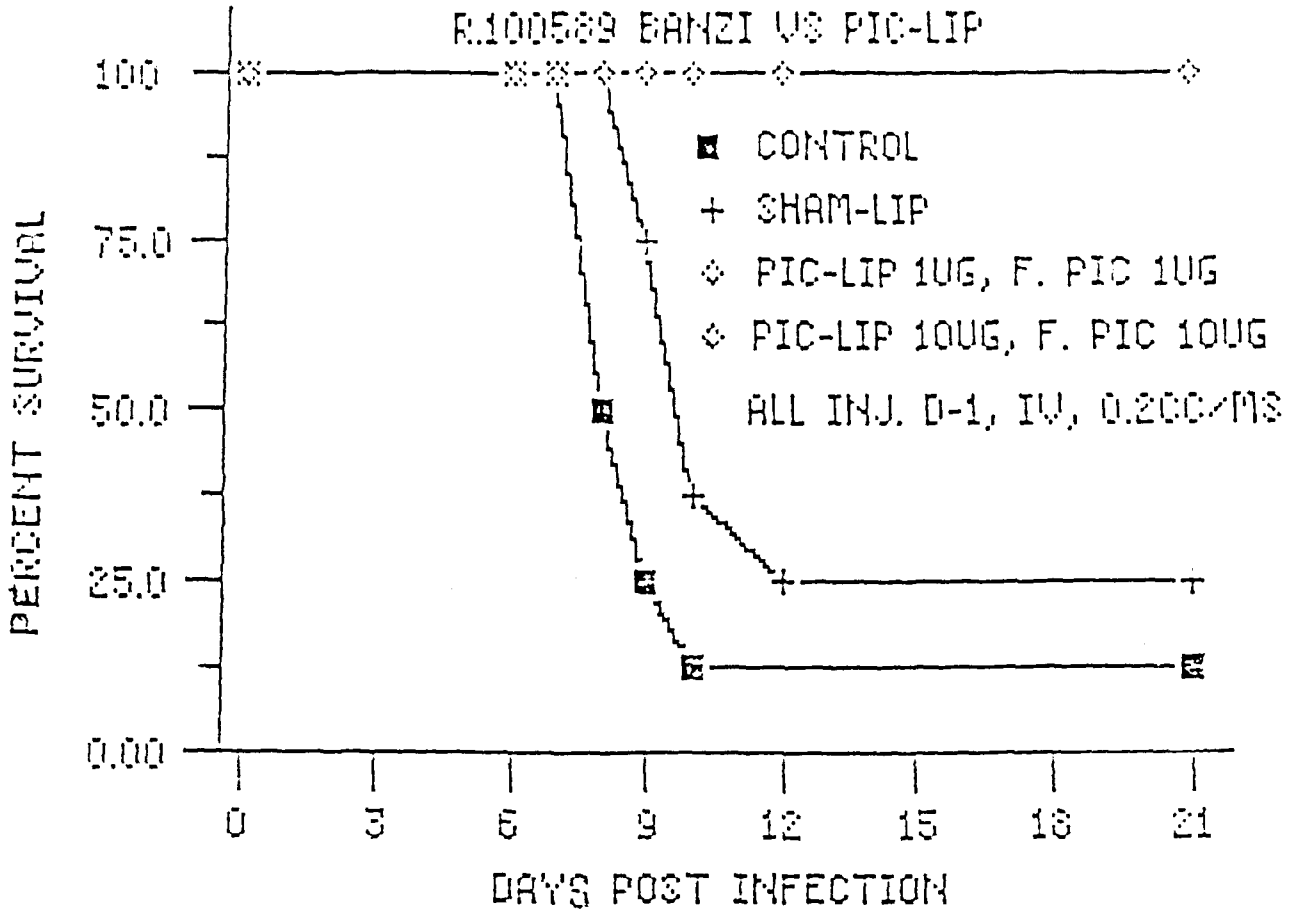


Figure 19. Augmentation of resistance to Banzi virus by liposomal or free Poly I:C administered one day prior to infection.

Six-week-old C3H/Hen mice were intravenously inoculated with either free or liposome-encapsulated poly I:C (50 or 500 μg/kg) one day prior to challenge with 1 LD80 of Banzi virus. Note that <> represents both free and liposomal drug at 50 and 500 μg/kg.

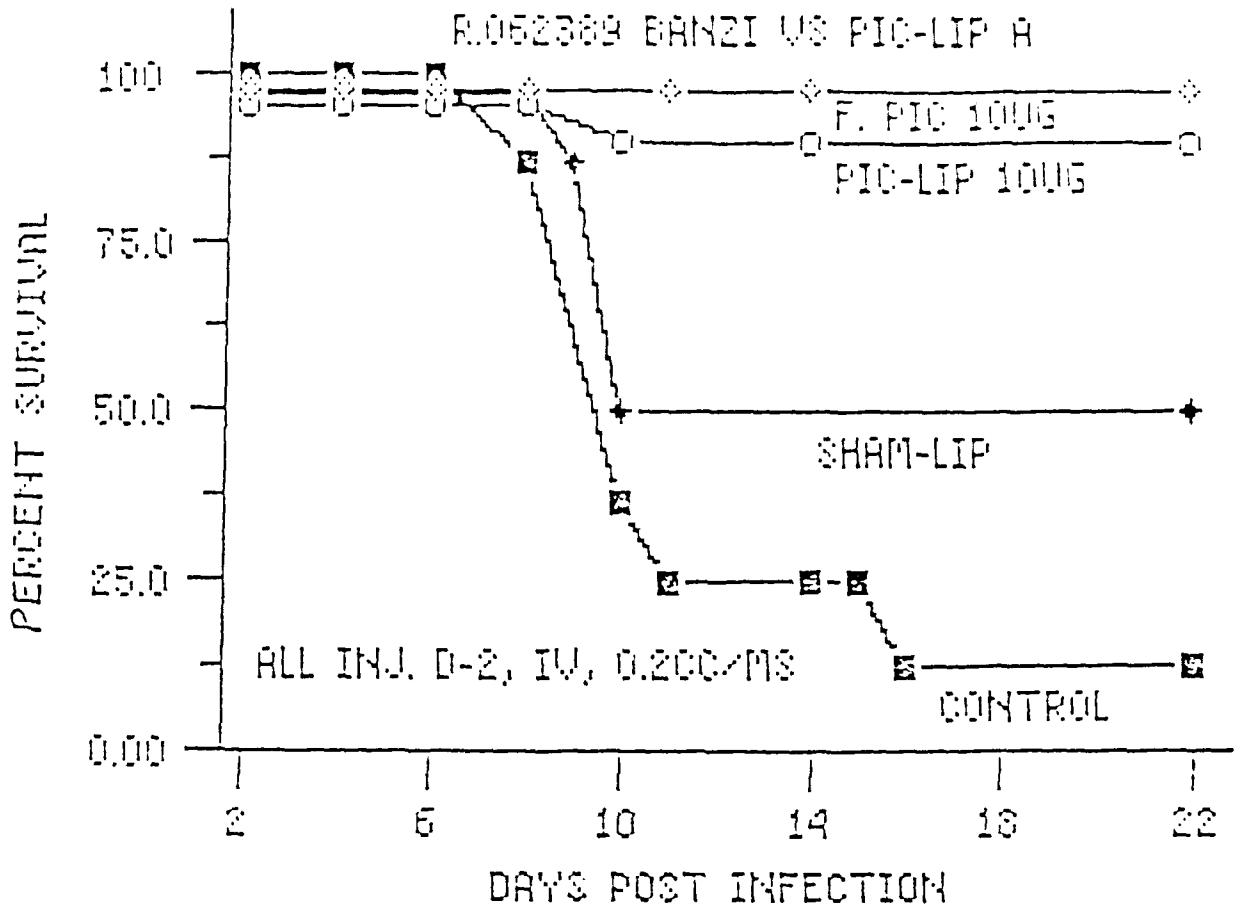


Figure 20. Augmentation of resistance to Banzi virus by liposomal or free Poly I:C administered two days prior to infection.

Six-week-old C3H/Hen mice were intravenously inoculated with either free or liposome-encapsulated Poly I:C (500 μg/kg) two days prior to challenge with 1 LD80 of Banzi virus.

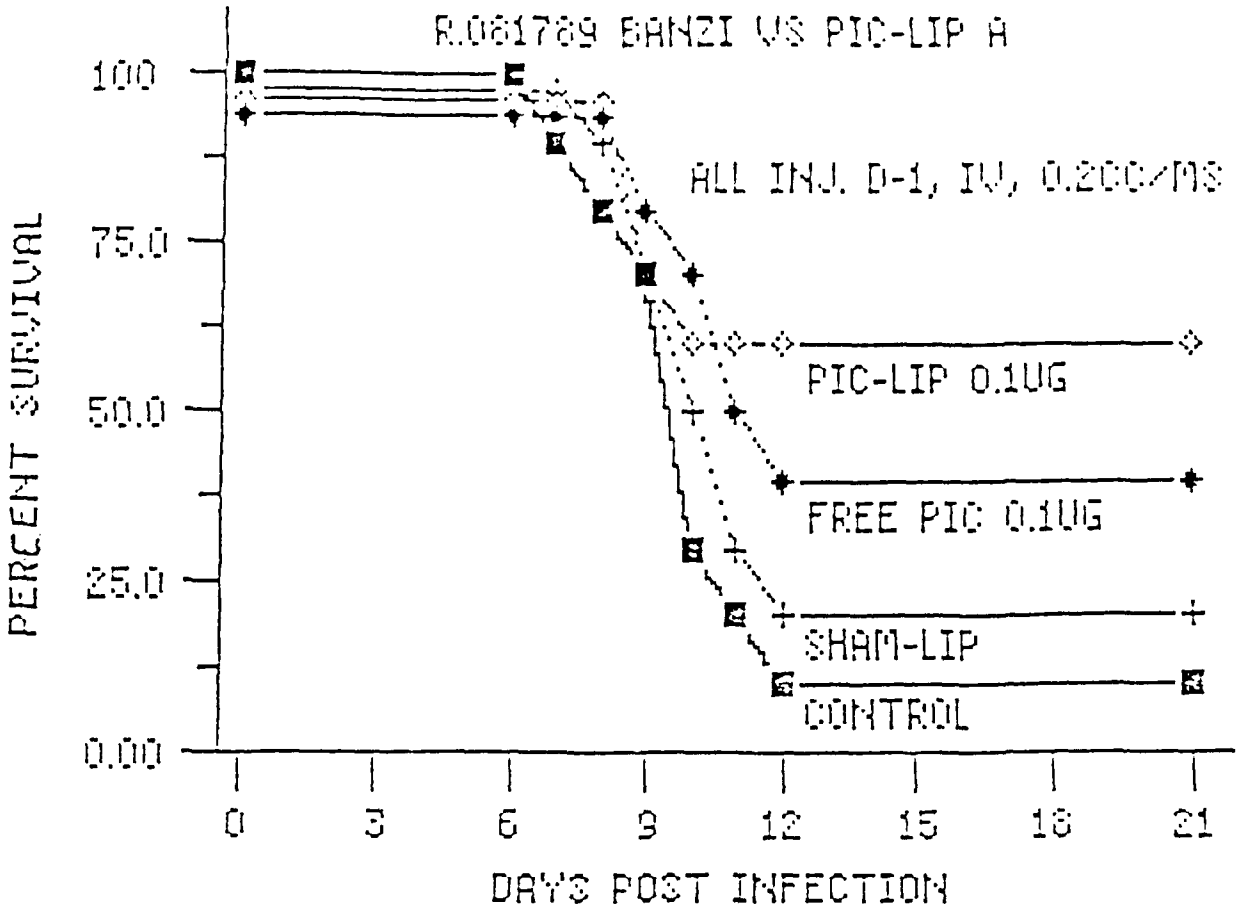


Figure 21. Augmentation of resistance to Banzi virus by low dose liposomal or free Poly I:C administered one day prior to infection.

Six-week-old C3H/Hen mice were intravenously inoculated with either free or liposome-encapsulated Poly I:C (5 μg/kg) one day prior to challenge with 1 LD80 of Banzi virus.

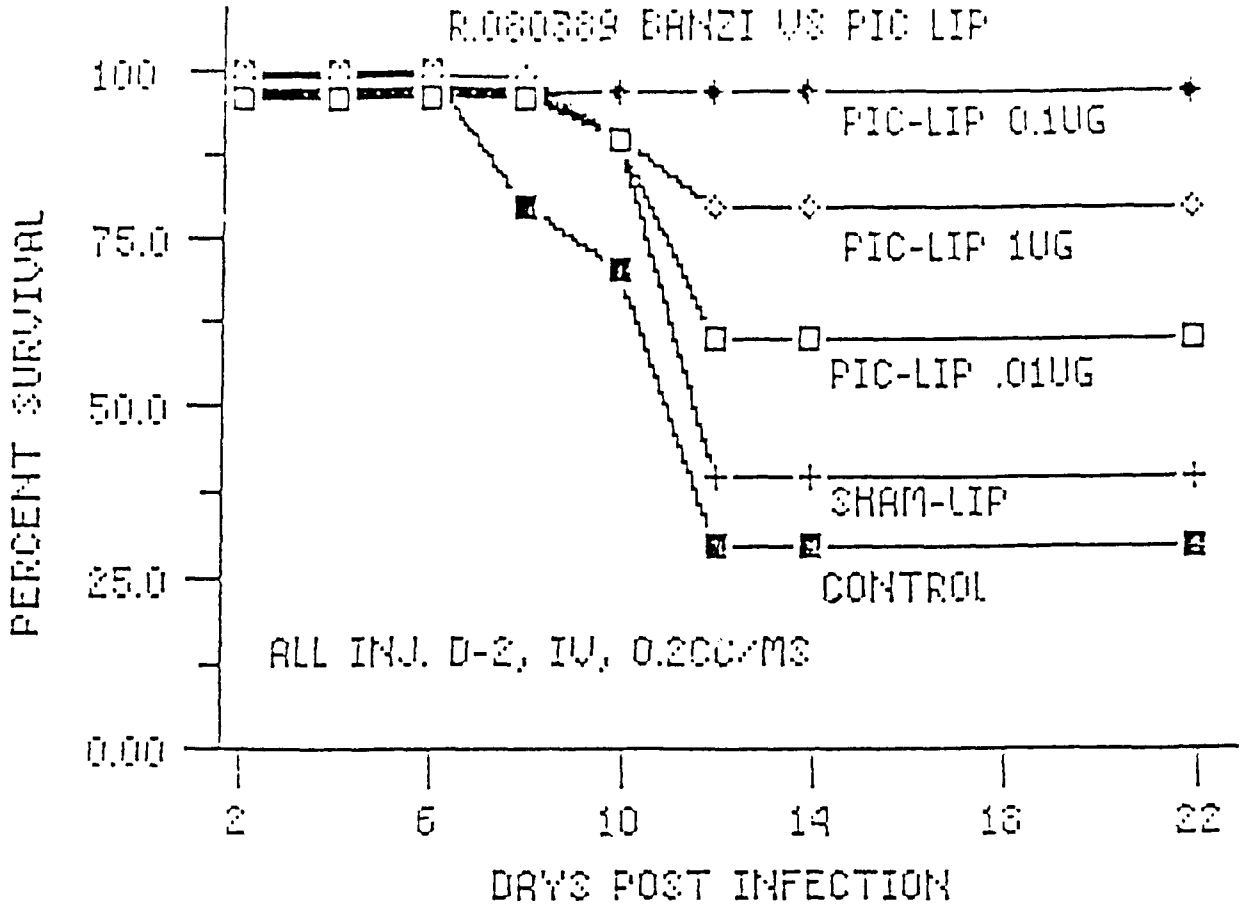
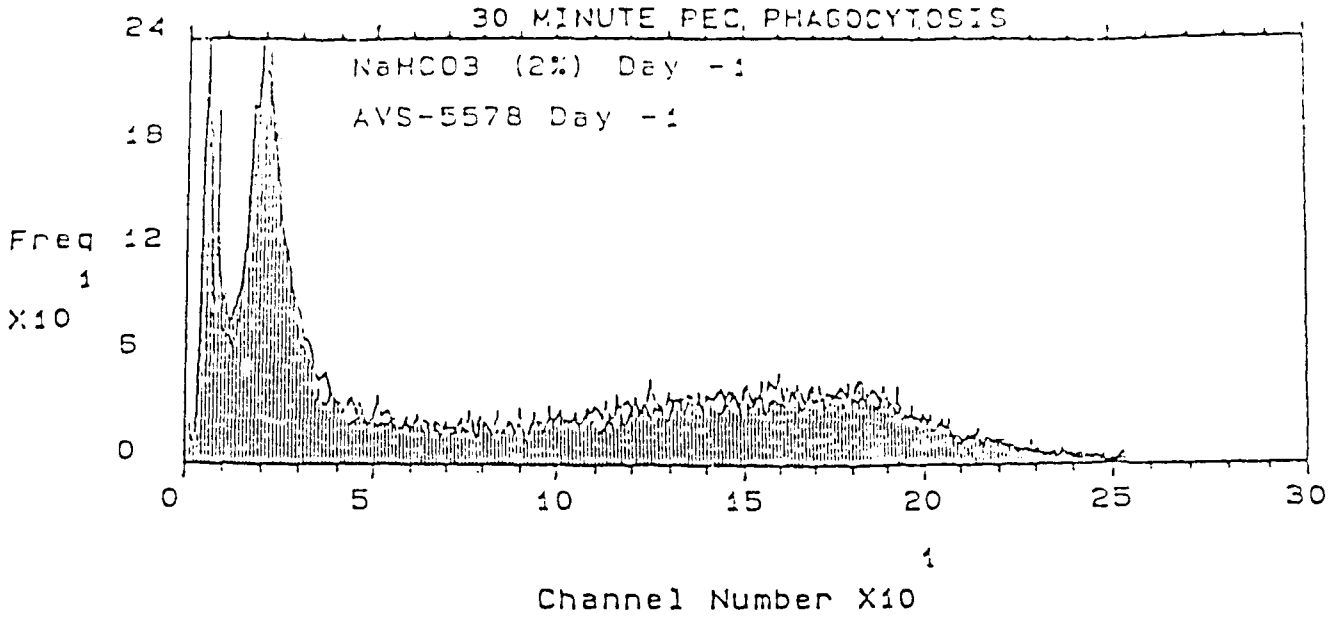


Figure 22. Augmentation of resistance to Banzi virus by low dose liposomal Poly I:C administered two days prior to infection.

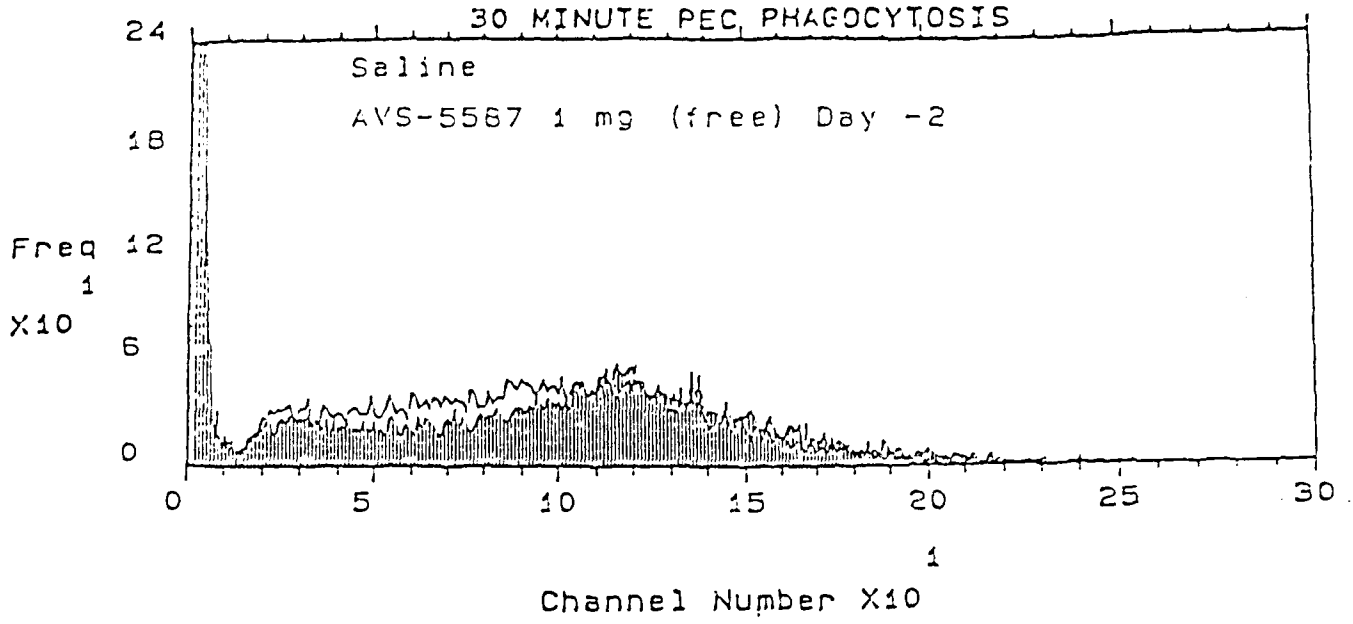
Six-week-old C3H/Hen mice were intravenously inoculated with liposome-encapsulated Poly I:C (50, 5, or 0.5 μg/kg) two days prior to challenge with 1 LD80 of Banzi virus.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
2% $NaHCO_3$		49	5	26	18
AVS-5587		51	5	29	17

Figure 23. Phagocytosis by peritoneal exudate cells following oral administration of AVS-5587 on day -1.

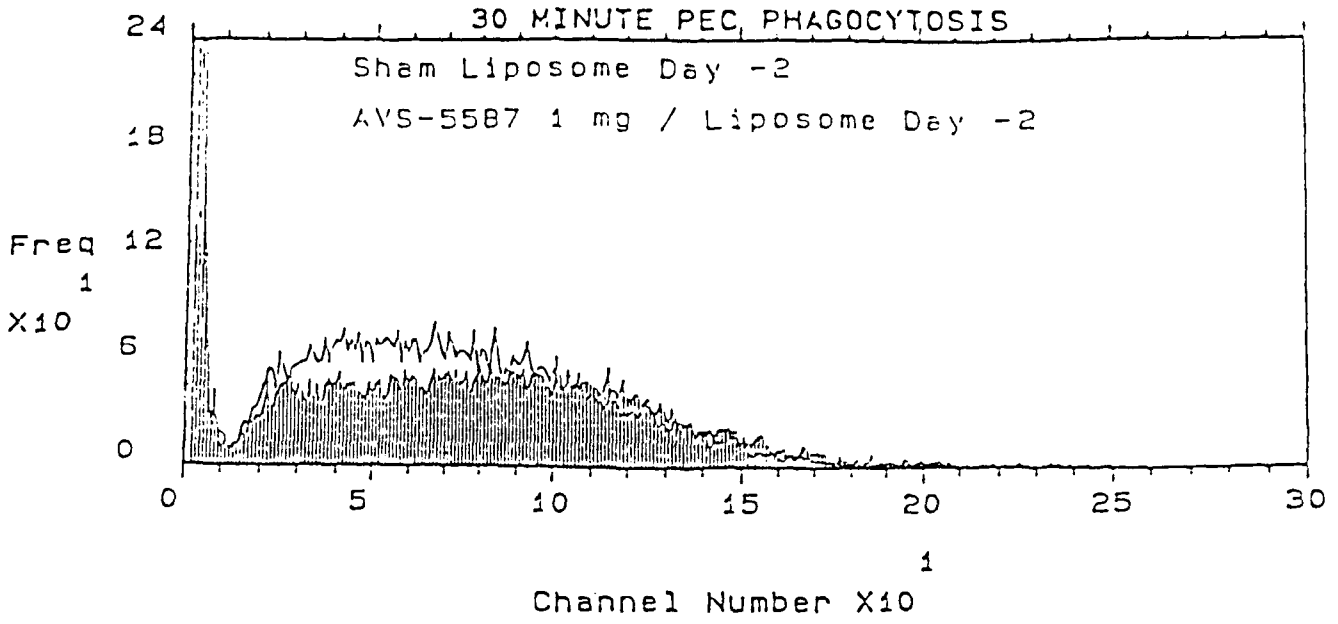
Mice were given AVS-5587 (10 mg/kg) in 2% $NaHCO_3$, orally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the $NaHCO_3$ control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
$NaHCO_3$		43	7	30	6
AVS-5587		41	9	29	3

Figure 24. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of AVS-5587 on day -2.

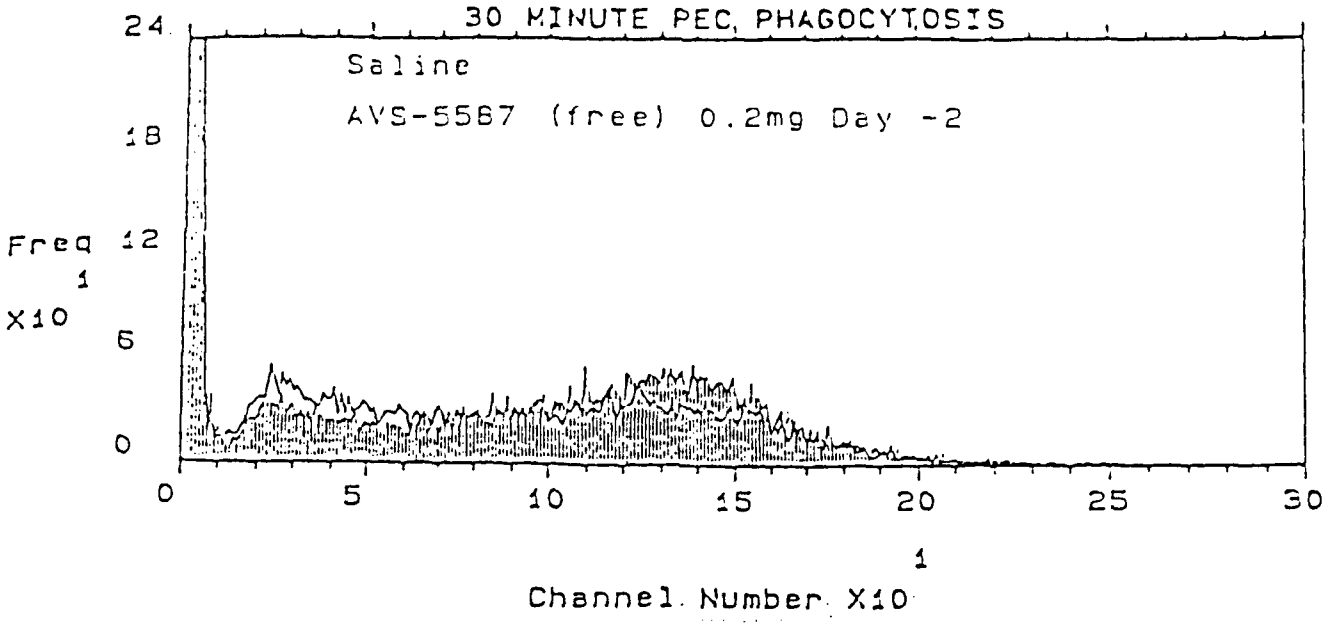
Mice were given AVS-5587 (10 mg/kg) in 2% $NaHCO_3$, intra- peritoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the $NaHCO_3$ control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Sham Liposomes		42	12	27	2
AVS-5587/Liposomes		43	17	25	1

Figure 25. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated AVS-5587 on day -2.

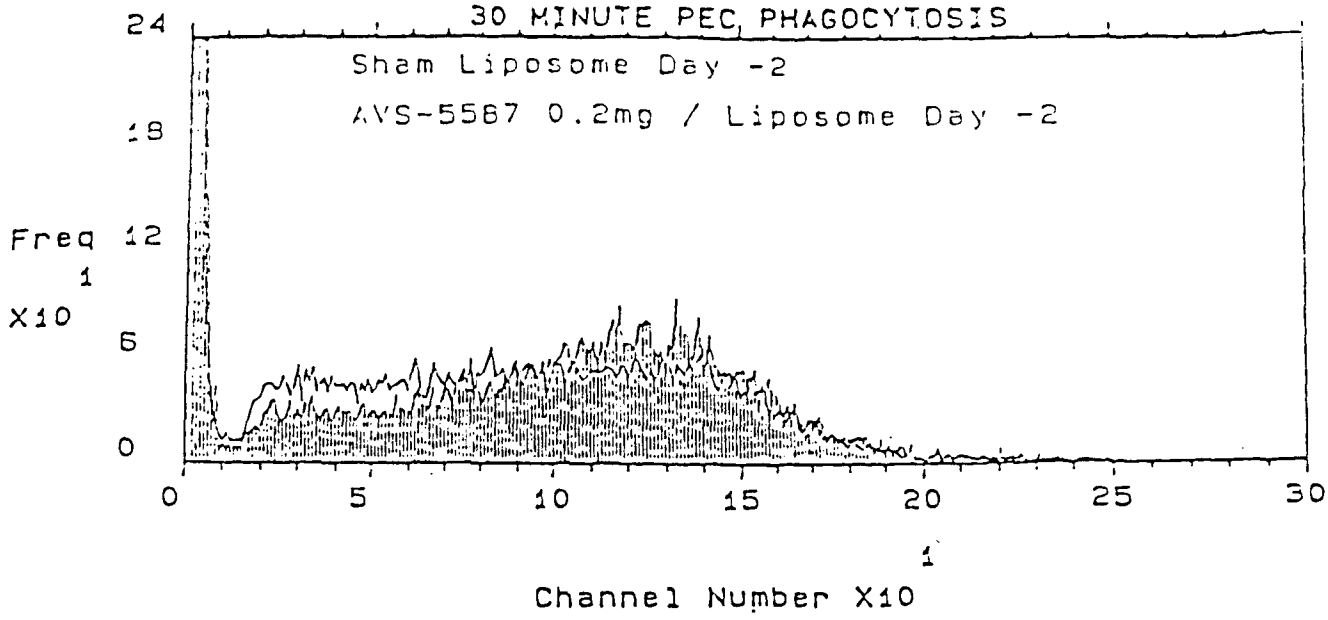
Mice were given liposome encapsulated AVS-5587 (10 mg/kg) in 2% $NaHCO_3$, intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the sham liposome control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
$NaHCO_3$		44	6	33	5
AVS-5587		37	7	26	4

Figure 26. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of AVS-5587 on day -2.

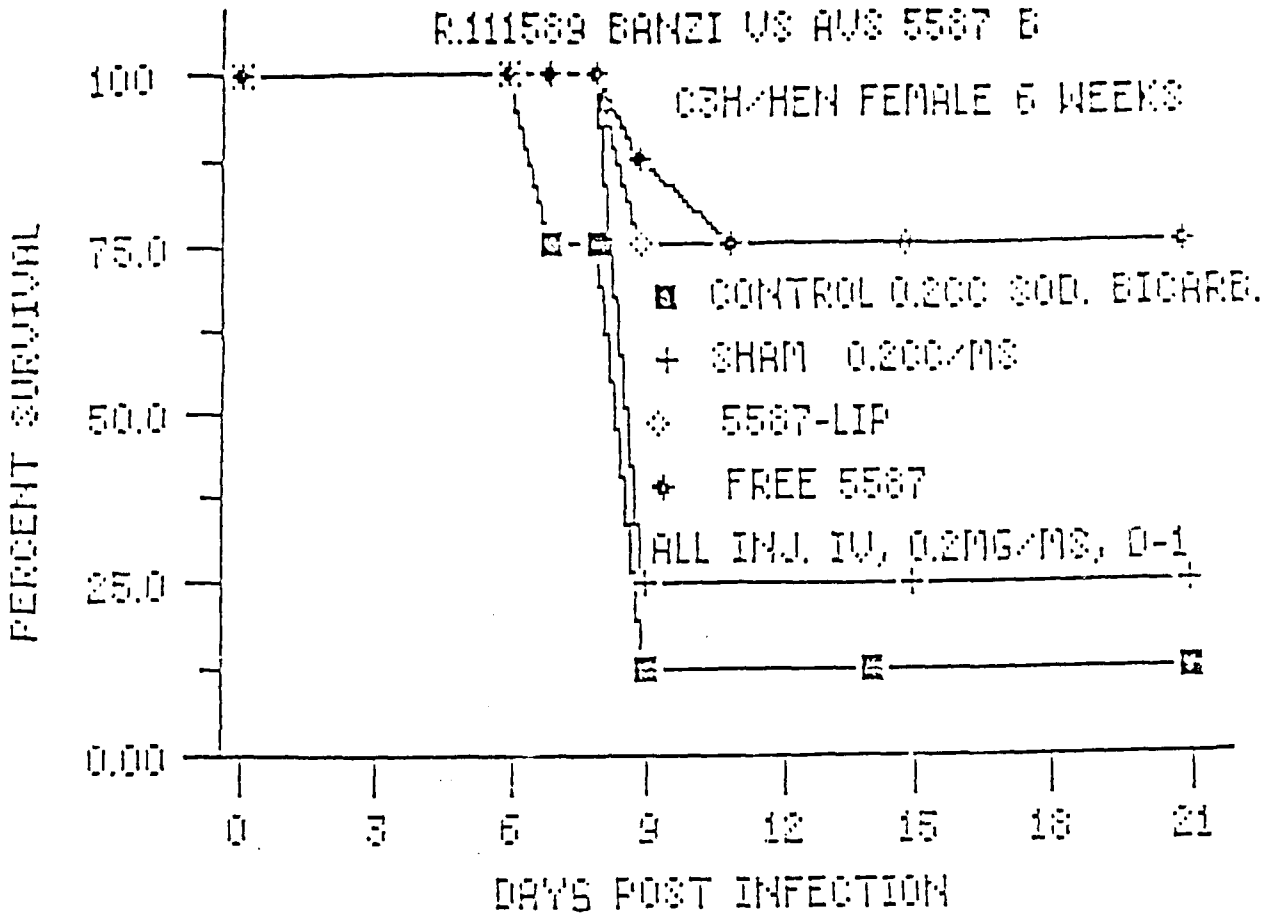
Mice were given AVS-5587 (50 mg/kg) in 2% $NaHCO_3$, intra- peritoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the $NaHCO_3$ control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Sham Liposomes		64	10	48	6
AVS-5587/Liposomes		57	13	41	4

Figure 27. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated AVS-5587 on day -2.

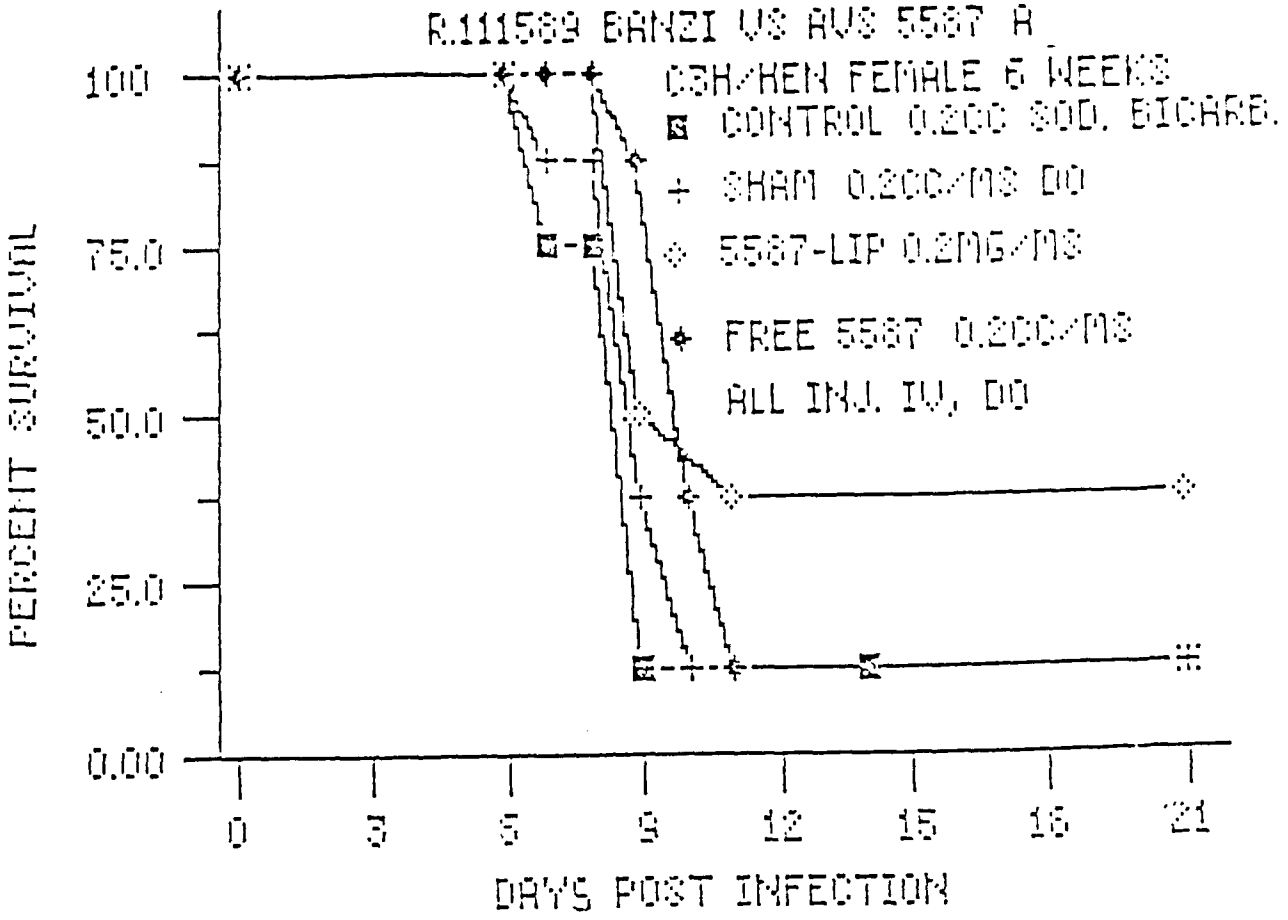
Mice were given liposome encapsulated AVS-5587 (50 mg/kg) in 2% $NaHCO_3$, intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the sham liposome control.



Treatment	MST (Days)	P Value
Control	9.40	-
Sham Liposomes	11.12	NS
AVS-5587/Liposomes	16.99	<0.05
Free AVS-5587	17.22	<0.05

Figure 28. Augmentation of resistance to Banzi virus by liposomal or free AVS-5587 administered one day prior to infection.

Six week old C_3H/HeN mice were intravenously inoculated with either free or liposome-encapsulated AVS-5587 (10 mg/kg) one day prior to challenge with 1 LD_{80} of Banzi virus.



Treatment	MST (Days)	P Value
Control	9.40	-
Sham Liposomes	9.95	NS
AVS-5587/Liposomes	12.68	NS
Free AVS-5587	10.34	NS

Figure 29. Augmentation of resistance to Banzi virus by liposomal or free AVS-5587 administered on the day of virus infection.

Six week old C_3H/HeN mice were intravenously inoculated with either free or liposome-encapsulated AVS-5587 (10 mg/kg) on the day of challenge with 1 LD_{80} of Banzi virus.

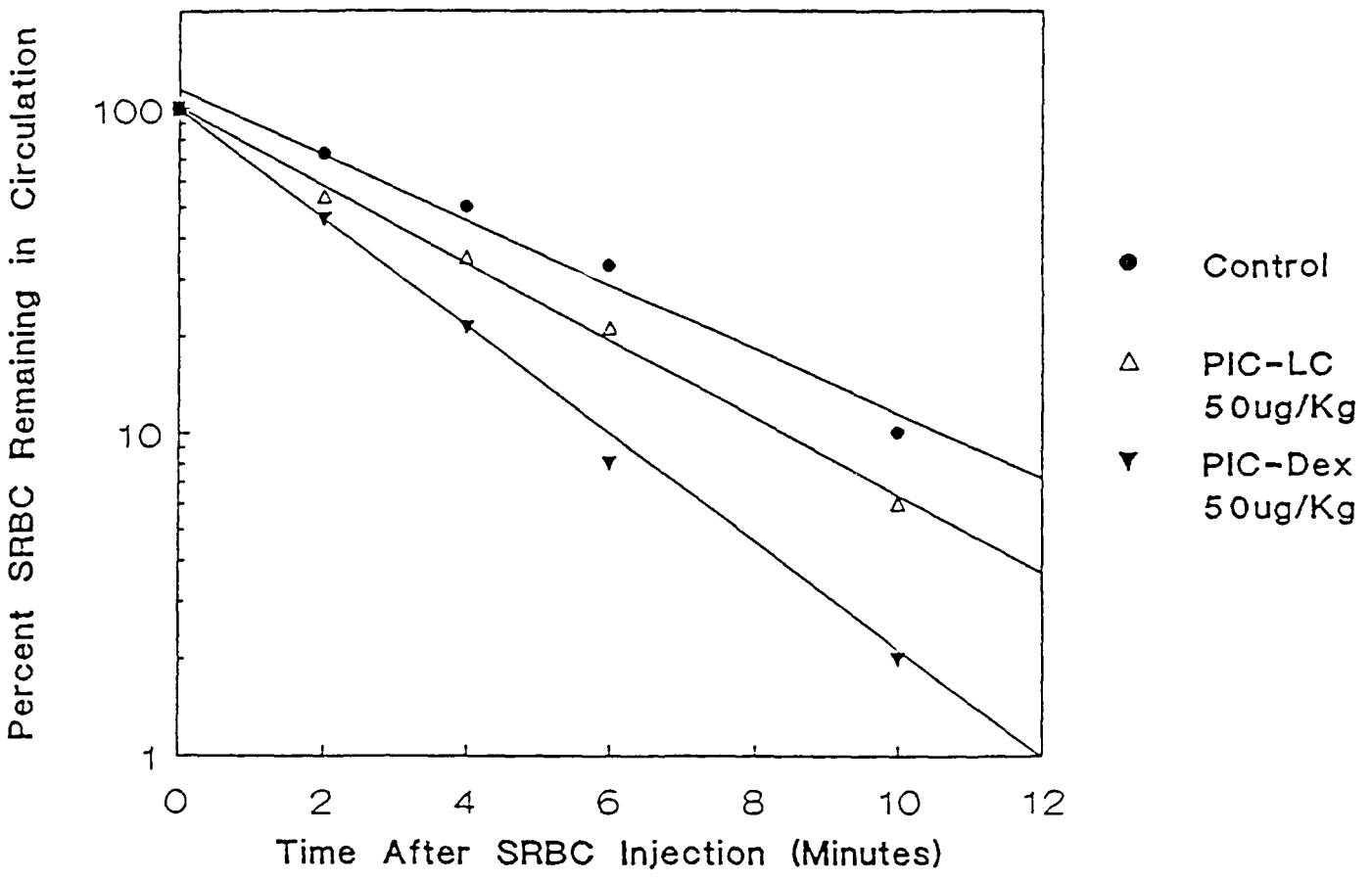


Figure 30. Effect of Poly I:C-LC and Poly I:C-Dex on the RES function (D-1, IV).

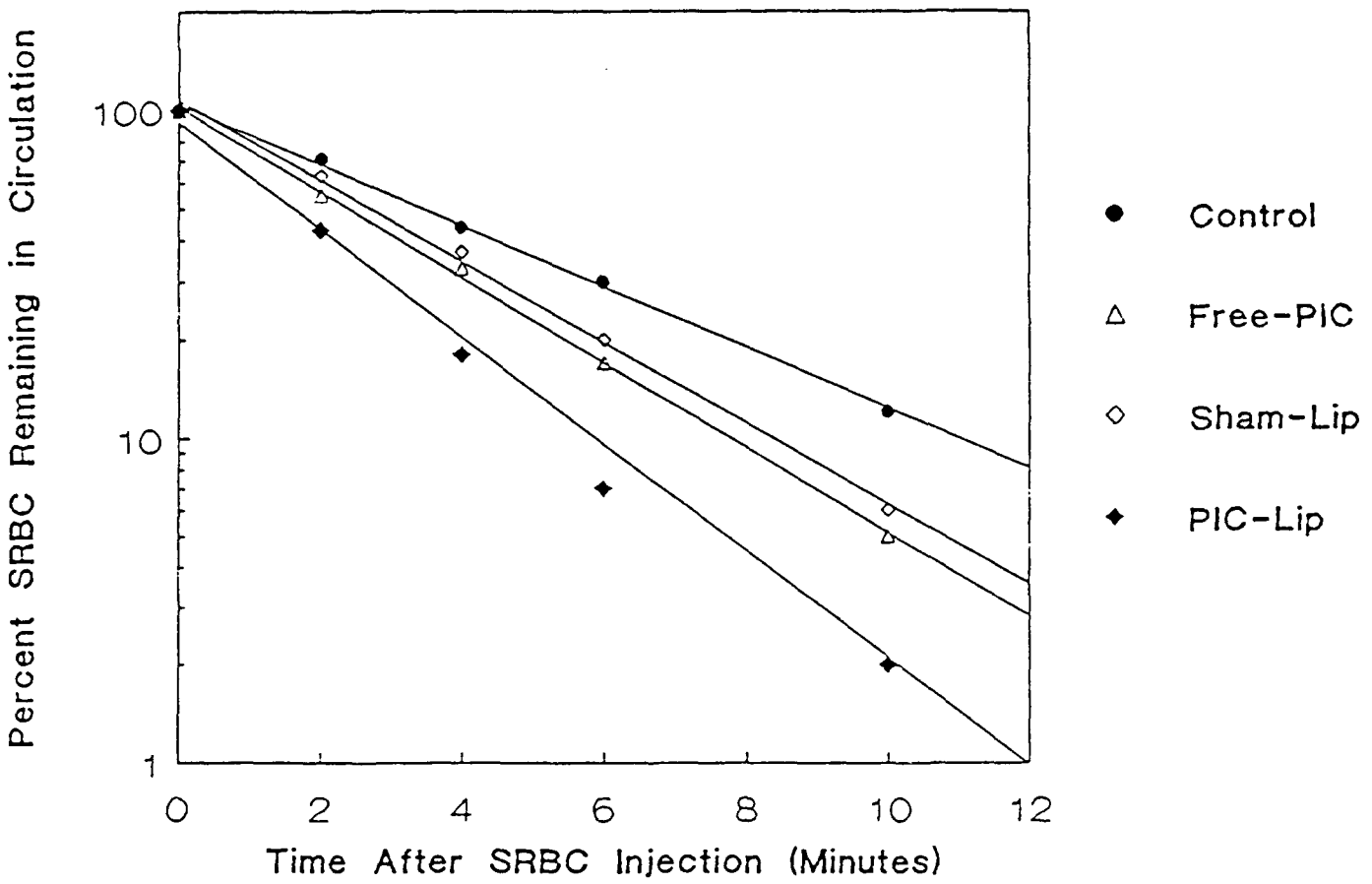
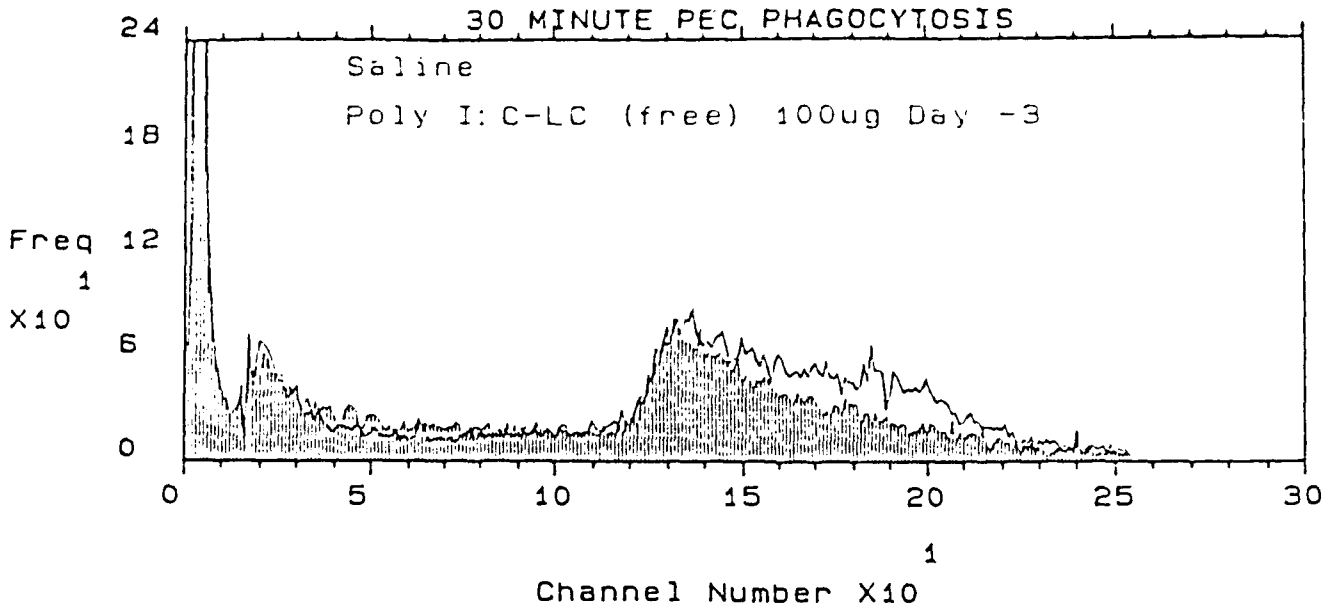


Figure 31. Effect of free or liposome-encapsulated Poly I:C on the RES function.

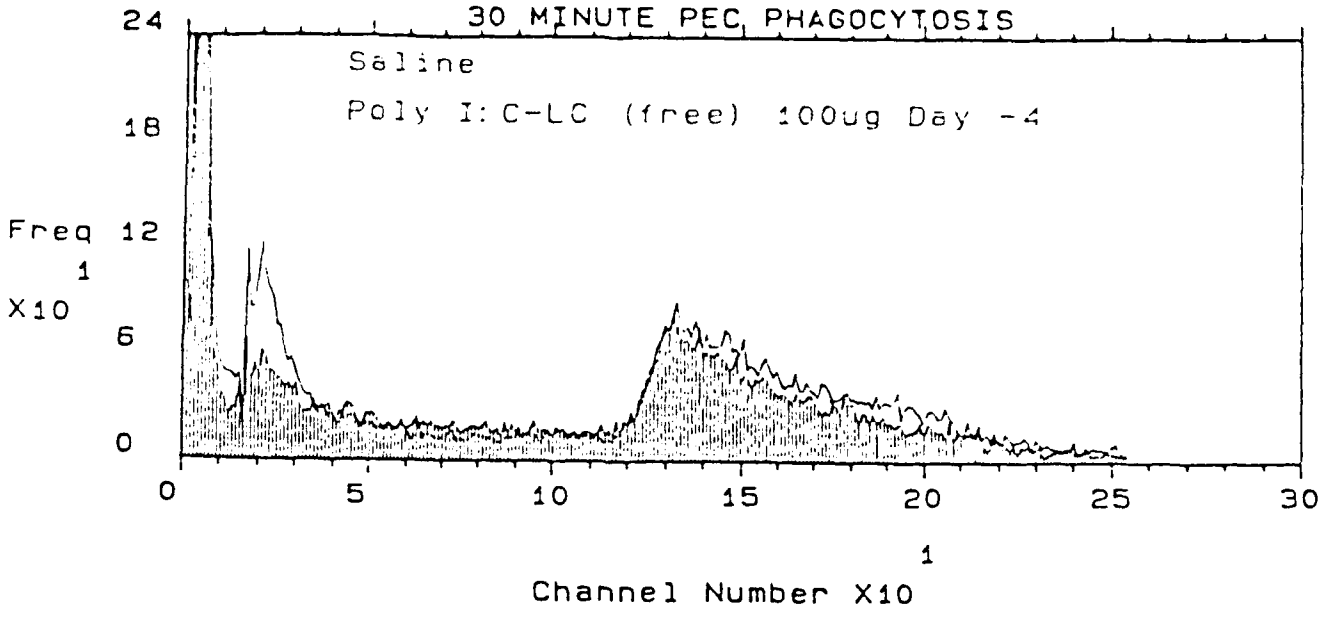


Treatment	Channel No.	% Phagocytic Cells Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Control		52	5	32	15
Poly I:C-LC		66	4	37	25*

Figure 32. Phagocytosis by peritoneal exudate cells following intravenous administration of free Poly I:C-LC on day -3.

Mice were given Poly I:C-LC (5 mg/kg), intravenously, three days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

* $p<0.01$

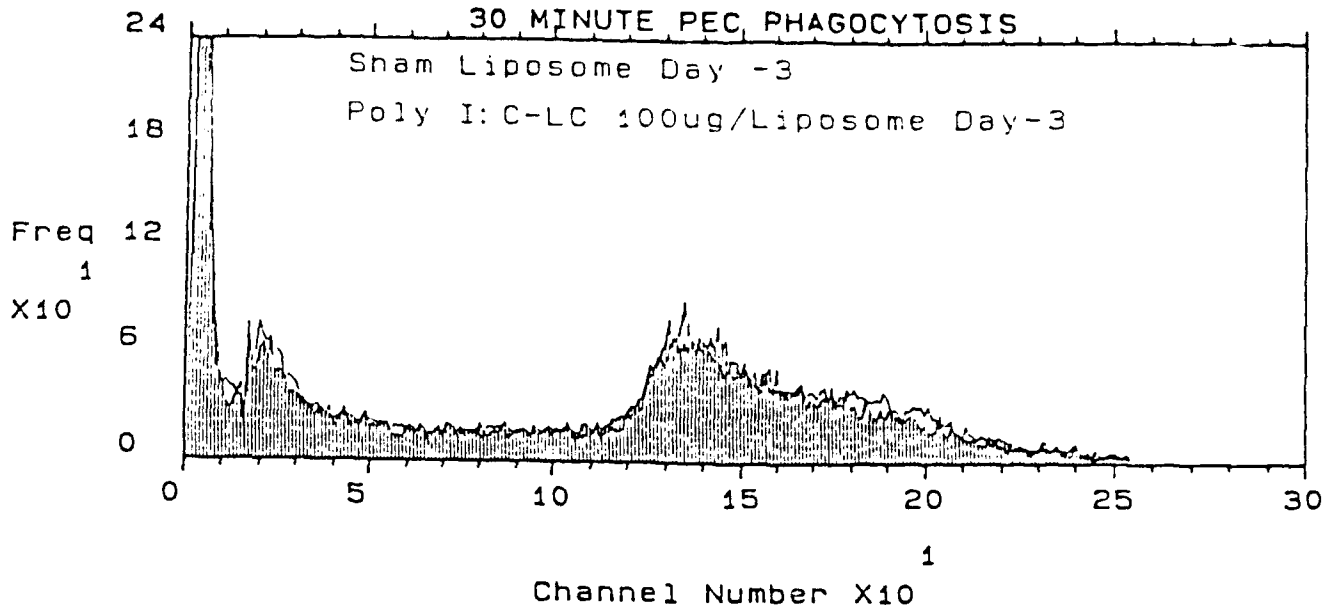


		% Phagocytic Cells			
Treatment	Channel No.	Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Control		52	5	32	15
Poly I:C-LC		57	3	34	20*

Figure 33. Phagocytosis by peritoneal exudate cells following intravenous administration of free Poly I:C-LC on day -4.

Mice were given Poly I:C-LC (5 mg/kg), intravenously, four days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

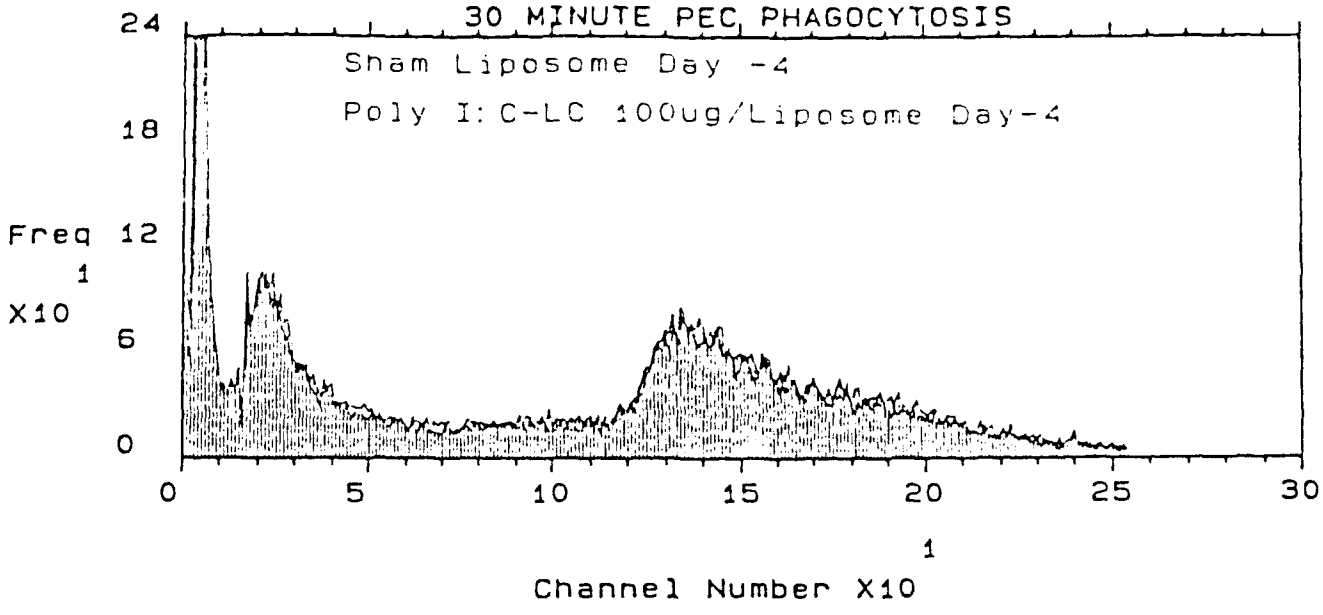
* $p<0.05$



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Sham Liposomes		56	4	35	17
Poly I:C-LC/Liposomes		53	4	31	18

Figure 34. Phagocytosis by peritoneal exudate cells following intravenous administration of liposome-encapsulated Poly I:C-LC on day -3.

Mice were given liposome-encapsulated Poly I:C-LC (5 mg/kg), intravenously, three days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Sham Liposomes		56	4	35	17
Poly I:C-LC/Liposomes		58	4	28	16

Figure 35. Phagocytosis by peritoneal exudate cells following intravenous administration of liposome-encapsulated Poly I:C-LC on day -4.

Mice were given liposome-encapsulated Poly I:C-LC (5 mg/kg), intravenously, four days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

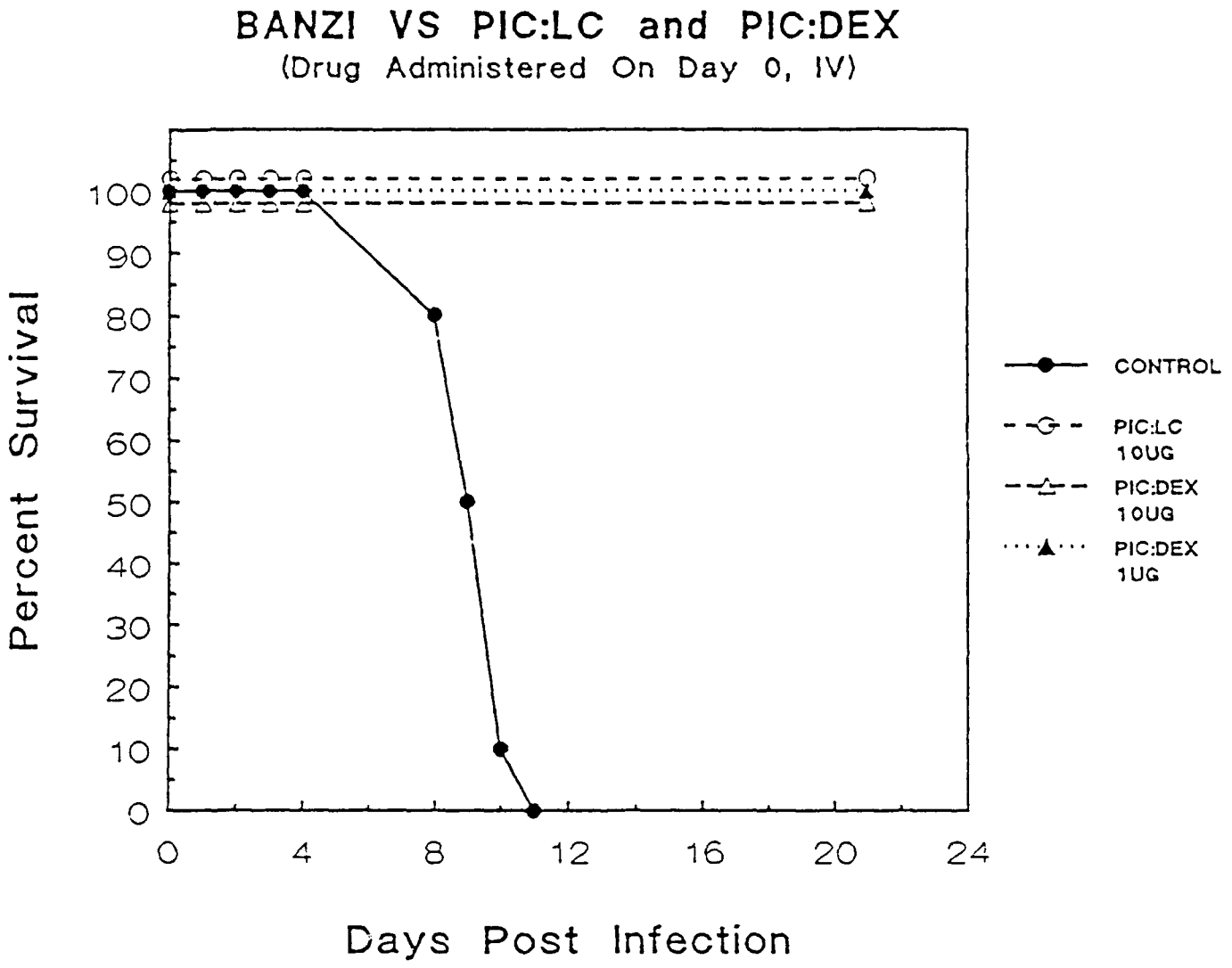


Figure 36. Effect of Poly I:C-LC (10 μg) and Poly I:C-Dex (10 μg), administered on day 0, on resistance to Banzi virus.

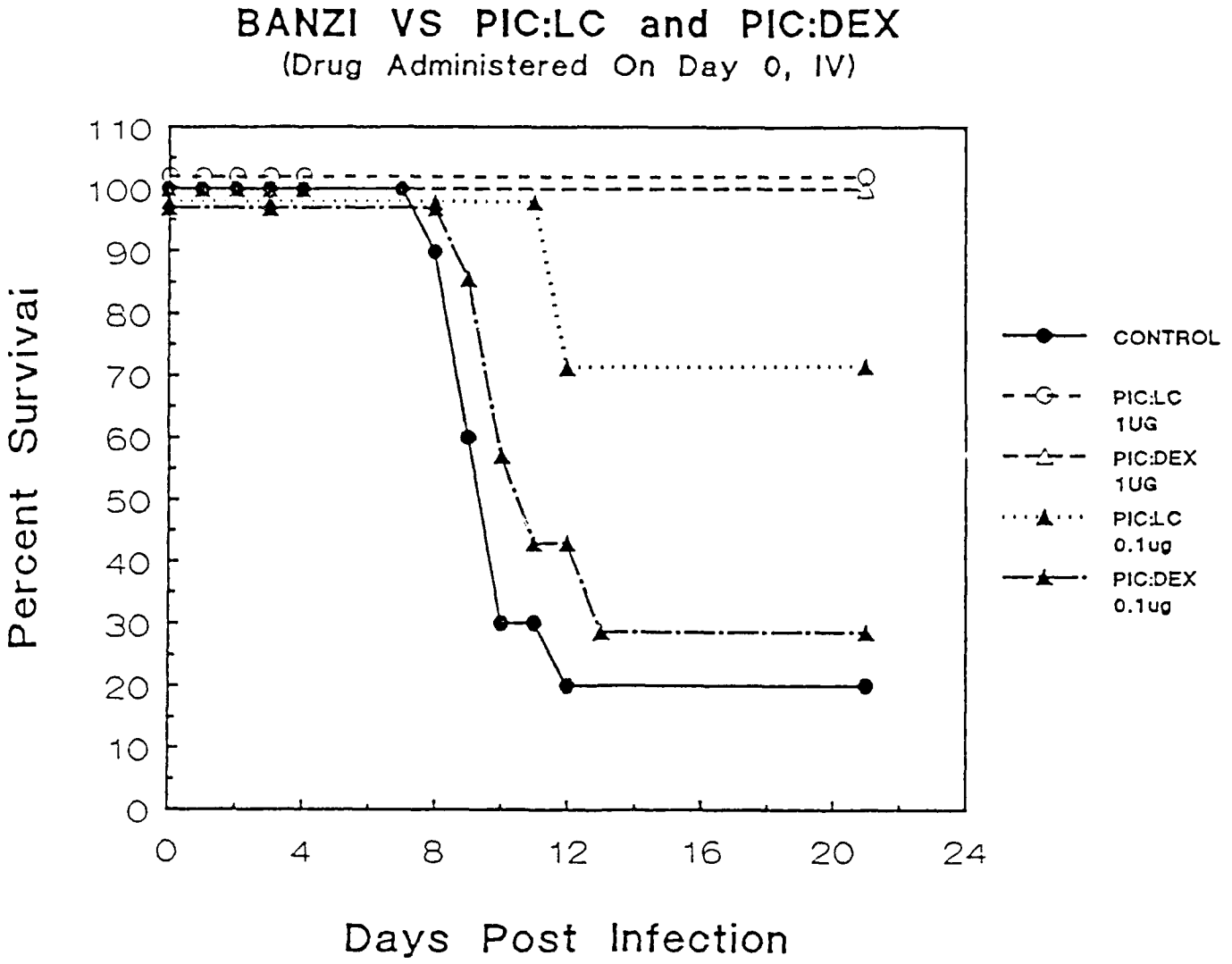


Figure 37. Effect of Poly I:C-LC (1 μg; 0.1 μg) and Poly I:C-Dex (1 μg; 0.1 μg), administered on day 0, on resistance to Banzi virus.

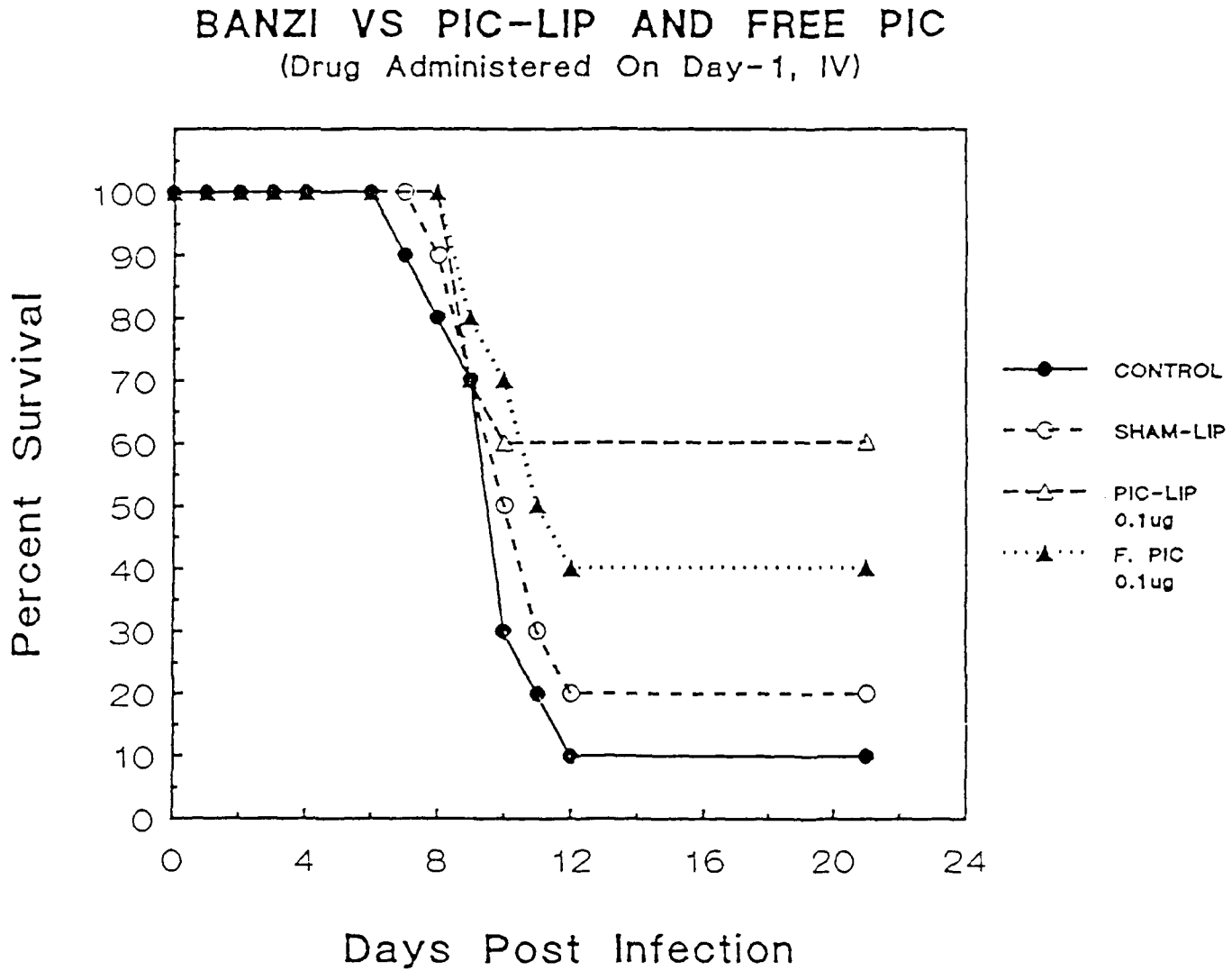


Figure 38. Effect of free and lysosome encapsulated Poly I:C, administered one day prior to infection, on resistance to Banzi virus.

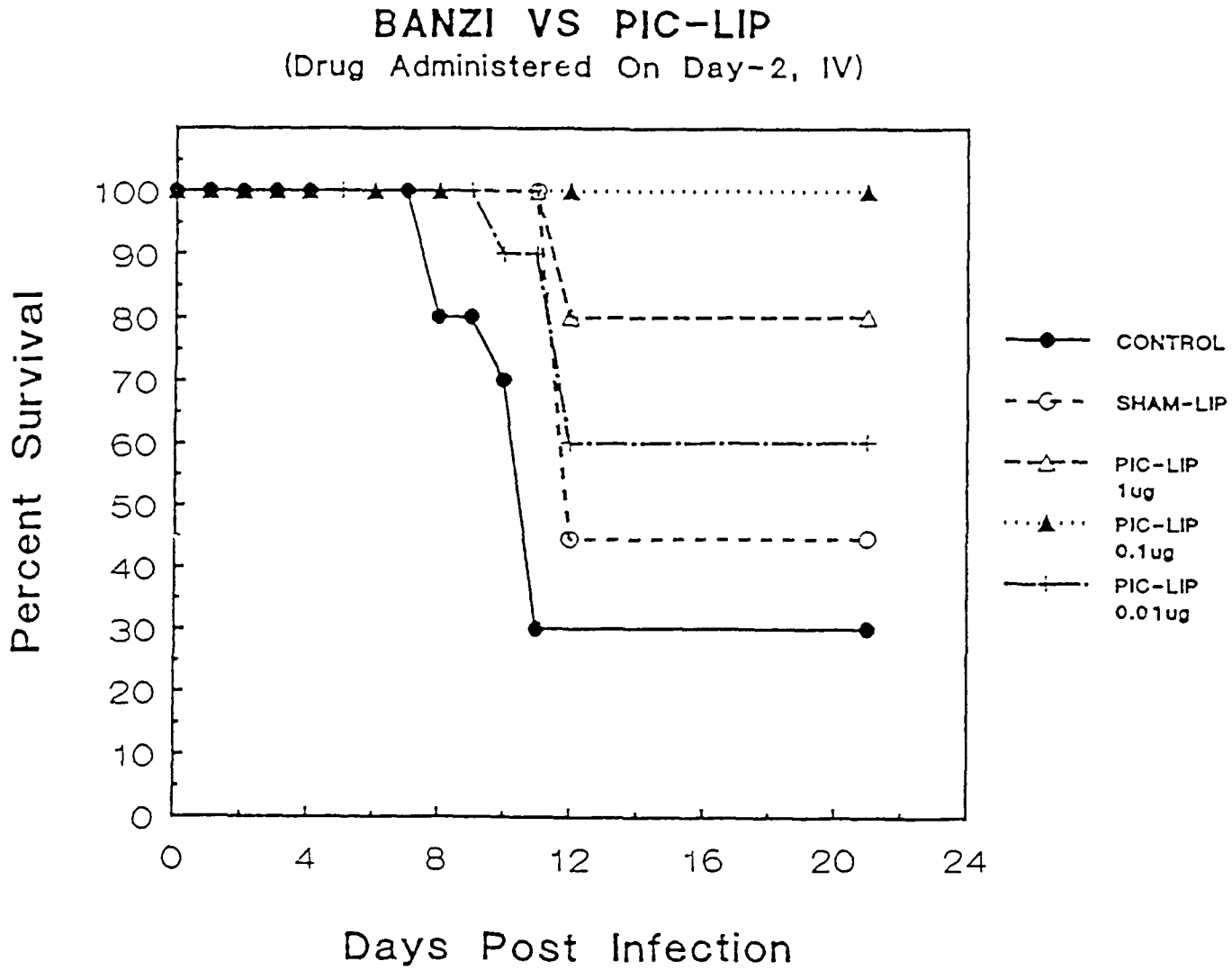
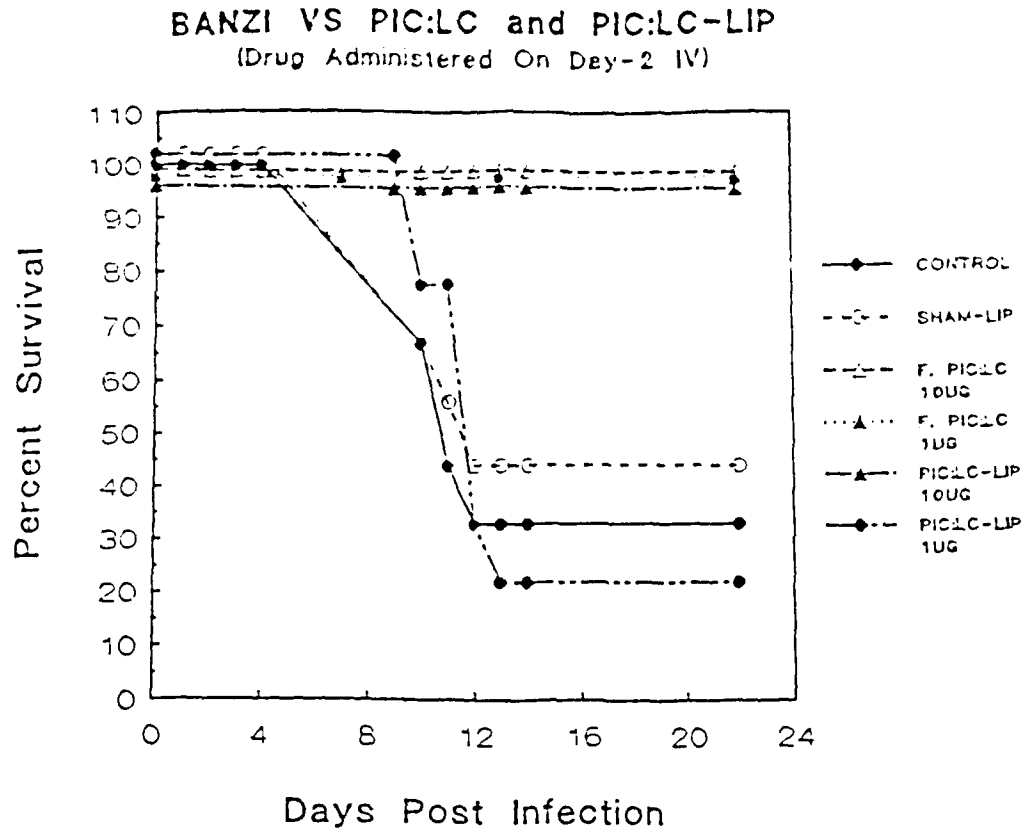


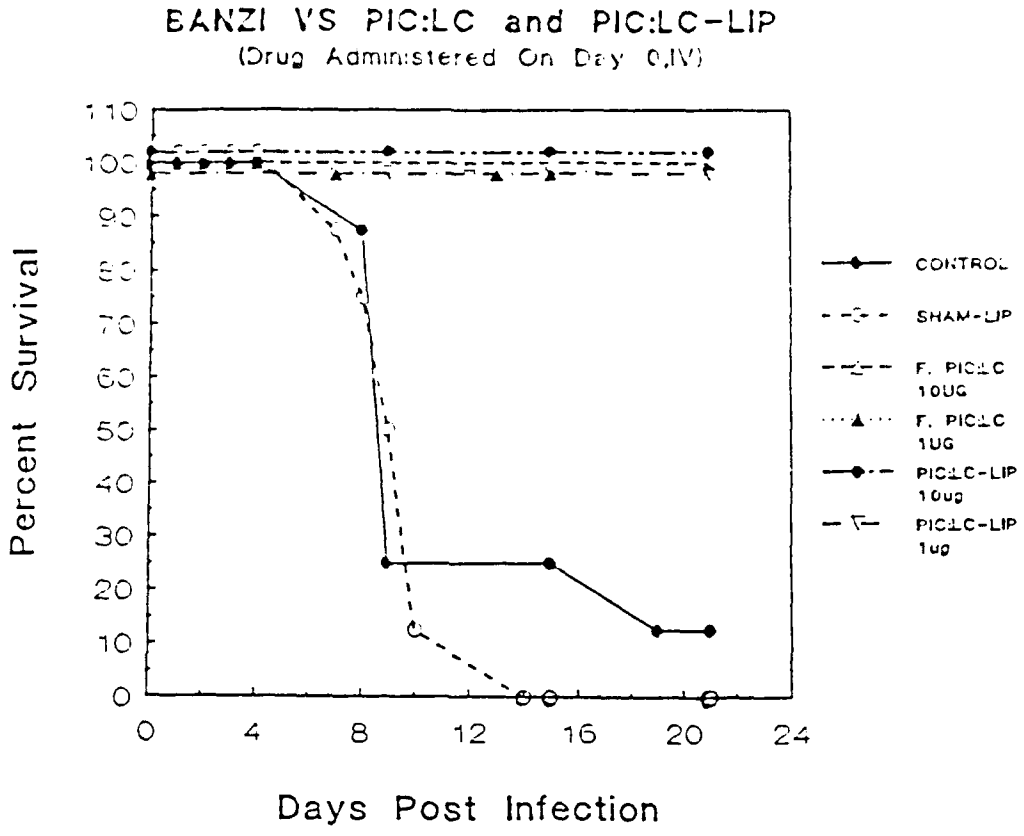
Figure 39. Effect of lysosome encapsulated Poly I:C, administered two days prior to infection, on resistance to Banzi virus.



Treatment	Geometric Mean Survival (Days)	P Value
Control	12.5	-
Sham Liposomes	14.3	NS
Free Poly I:C-LC 500 µg/kg	21.0	<0.001
Free Poly I:C-LC 50 µg/kg	21.0	<0.001
Liposome-encapsulated Poly I:C-LC 500 µg/kg	21.0	<0.001
Liposome-encapsulated Poly I:C-LC 50 µg/kg	13.3	NS

Figure 40. Augmentation of resistance to Banzi virus by free or liposome-encapsulated Poly I:C-LC administered two days prior to virus challenge.

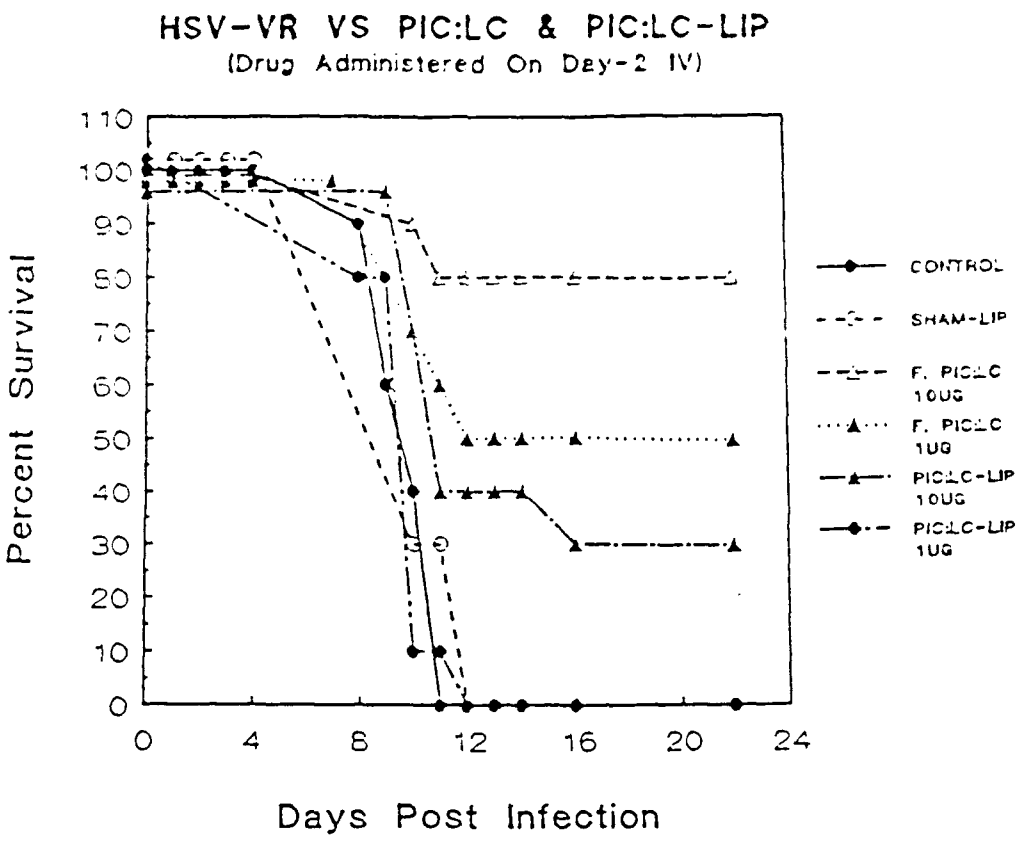
Six week old C_3H/HeN mice were intravenously inoculated with either free or liposome-encapsulated Poly I:C-LC two days prior to challenge with 1 LD_{80} of Banzi virus.



Treatment	Geometric Mean Survival (Days)	P Value
Control	10.89	-
Sham Liposomes	9.53	NS
Free Poly I:C-LC 500 μg/kg	22.00	<0.001
Free Poly I:C-LC 50 μg/kg	22.00	<0.001
Liposome-encapsulated Poly I:C-LC 500 μg/kg	22.00	<0.001
Liposome-encapsulated Poly I:C-LC 50 μg/kg	22.00	<0.001

Figure 41. Augmentation of resistance to Banzi virus by free or liposome-encapsulated Poly I:C-LC administered on the day of virus challenge.

Six week old C_3H/HeN mice were intravenously inoculated with either free or liposome-encapsulated Poly I:C-LC on the day of virus challenge with 1 LD_{80} of Banzi virus.



Treatment	Geometric Mean Survival (Days)	P Value
Control	9.8	-
Sham Liposomes	10.3	NS
Free Poly I:C-LC 500 μg/kg	18.3	<0.01
Free Poly I:C-LC 50 μg/kg	14.9	<0.005
Liposome-encapsulated Poly I:C-LC 500 μg/kg	13.9	<0.01
Liposome-encapsulated Poly I:C-LC 50 μg/kg	9.7	NS

Figure 42. Resistance to HSV-1 pneumonitis by free or liposome-encapsulated Poly I:C-LC administered two days prior to infection.

Four week old C_3H/HeN mice were intranasally inoculated with either free or liposome-encapsulated Poly I:C-LC two days prior to challenge with 1 LD_{80} of HSV-1.

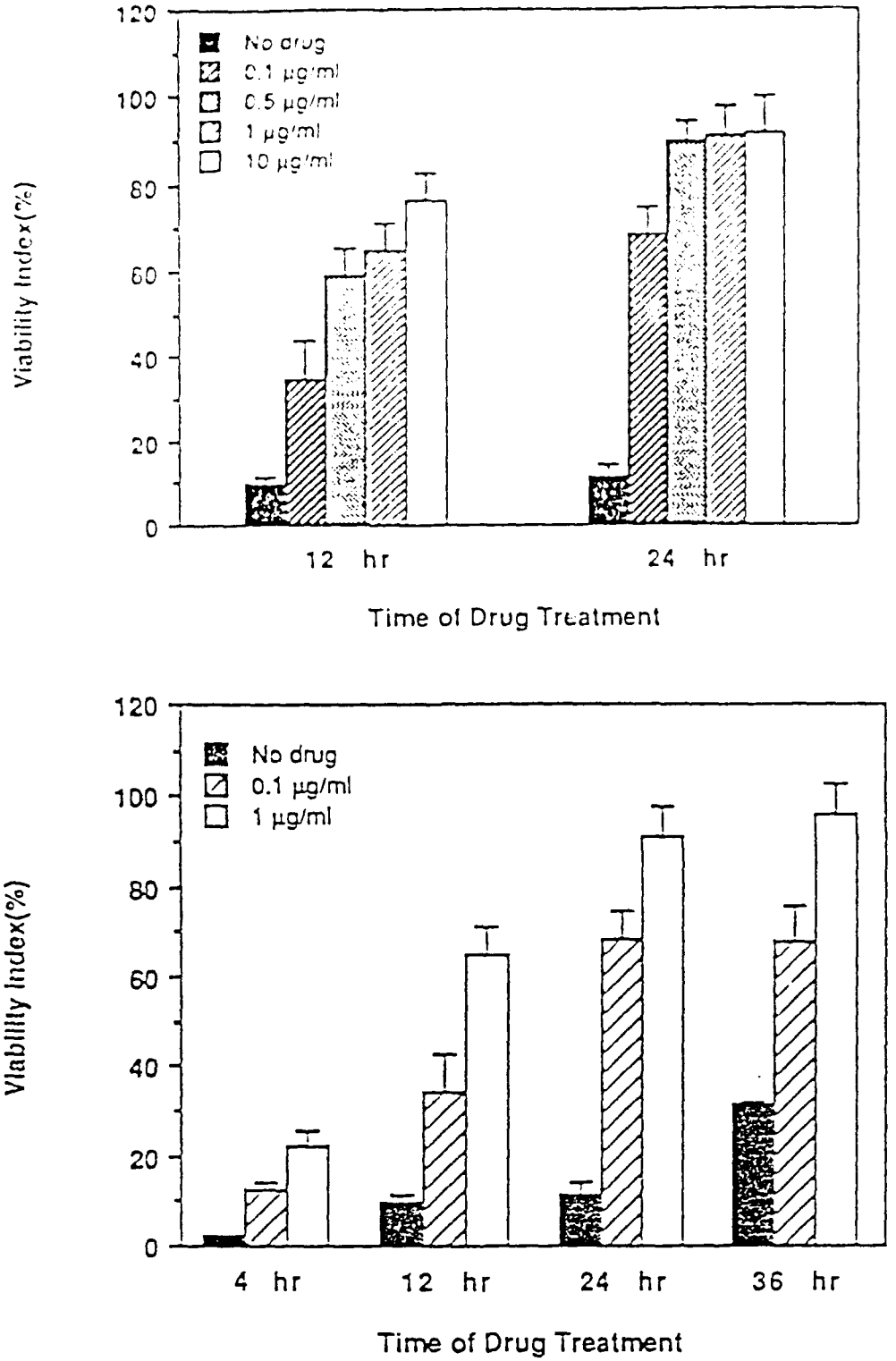


Figure 43. Kinetics and dose response of Poly I:C-induced antiviral activity.

Macrophages from CD-1 mice were treated *in vitro* with various doses of Poly I:C (3a) for various times (3b) before infection with HSV-1 (KOS strain: moi=7). CPE was assessed 48 hours later.

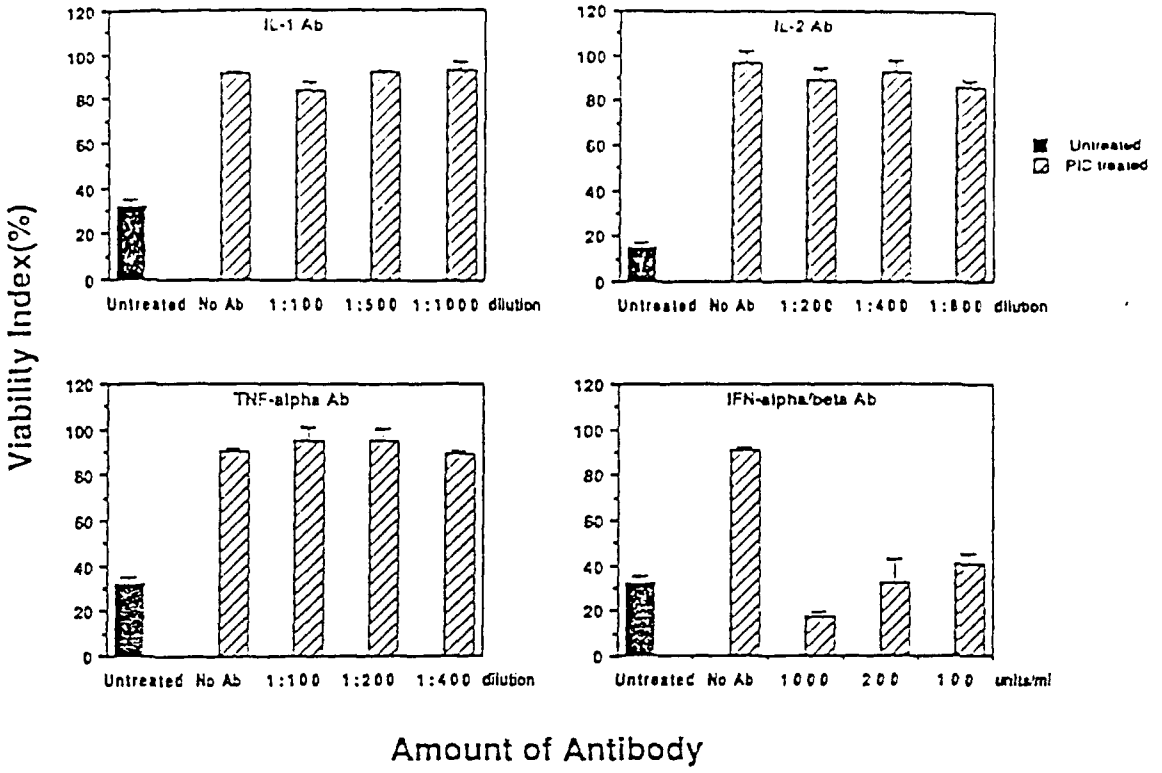


Figure 44. Neutralization of Poly I:C-induced antiviral activity by anti-IFN-α/β antibodies.

Macrophages were treated with Poly I:C (5 μg/ml) in the presence of various doses of antibodies to different cytokines. Cells were infected with HSV-1 (KOS strain; moi=7) 24 hours later and CPE determined after an additional 48 hours.

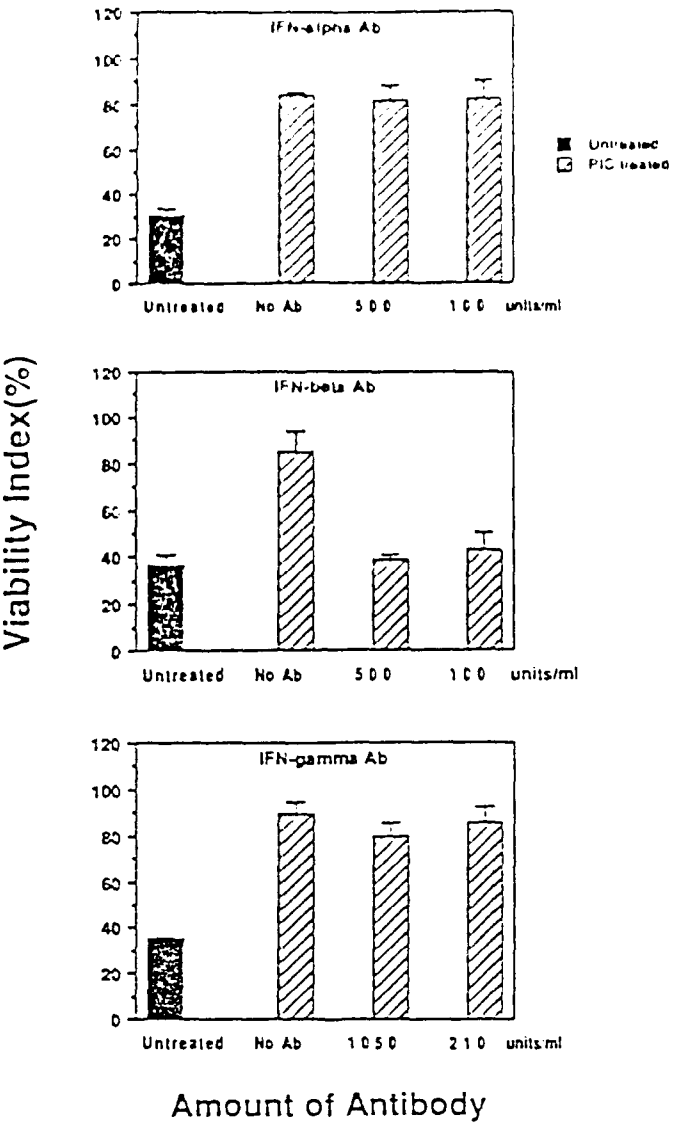


Figure 45. Neutralization of Poly I:C-induced antiviral activity by anti-IFN-α/β antibodies.

Macrophages were treated with Poly I:C (5 μg/ml) in the presence of various doses of antibodies to different interferon. Cells were infected with HSV-1 (KOS strain; moi=7) 24 hours later and cultured for an additional 48 hours in the presence of the antibodies. CPE was determined by the neutral red dye uptake assay.

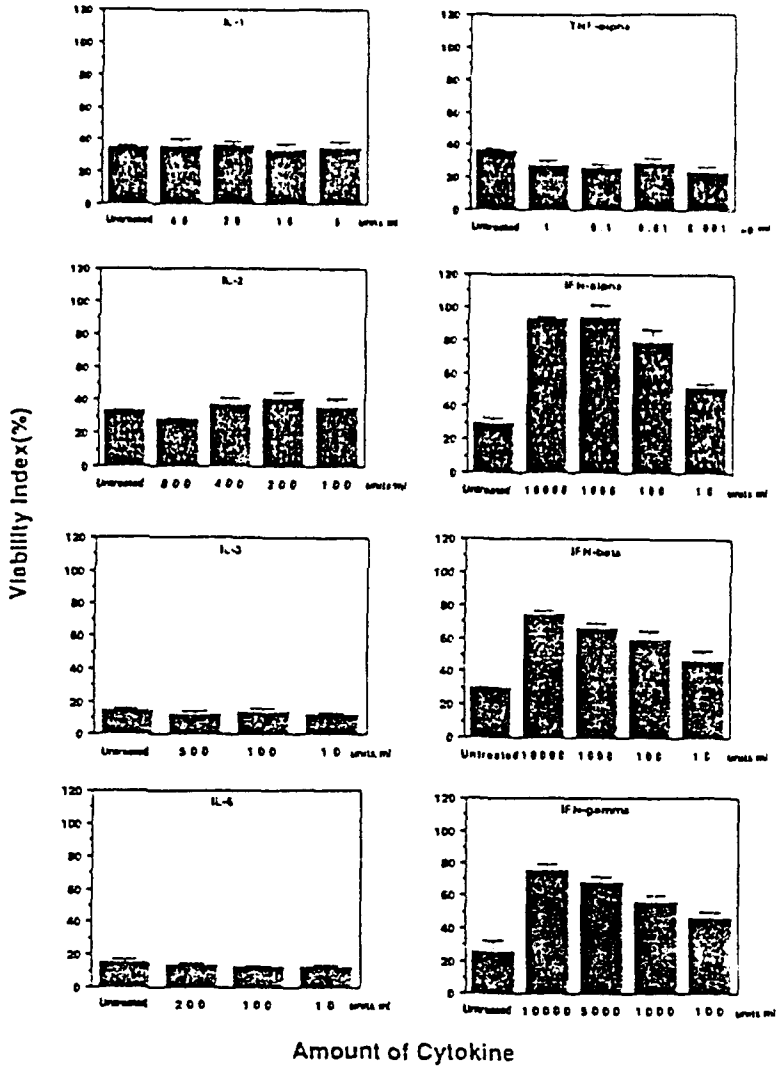


Figure 46. Antiviral activity of exogenous interferons.

Macrophages were preincubated with IFN-α, IFN-β or IFN-γ for 12 hours before infection with HSV-1 (KOS strain; moi=7). CPE was determined 48 hours later.

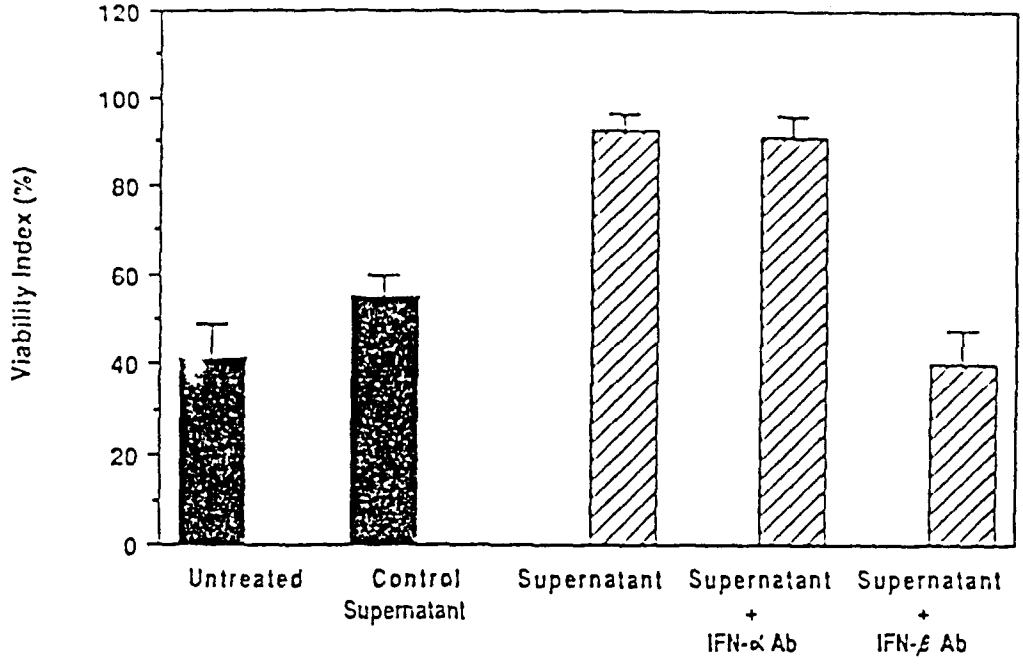


Figure 47. Detection of IFN-β in supernatants from Poly I:C treated macrophages.

Macrophages were treated with Poly I:C (5 μg/ml) for 6 hours and then washed twice. Controls were cultured with medium alone. After removal of Poly I:C, the cells were cultured for an additional 24 hours and supernatants were collected. Mouse fibroblasts (L929 cells) were treated for 12 hours with culture supernatant (50%) in the presence or absence of antibodies (500 units) to IFN-α or IFN-β. The cells were challenged with vesicular stomatitis virus and CPE assessed 48 hours later.

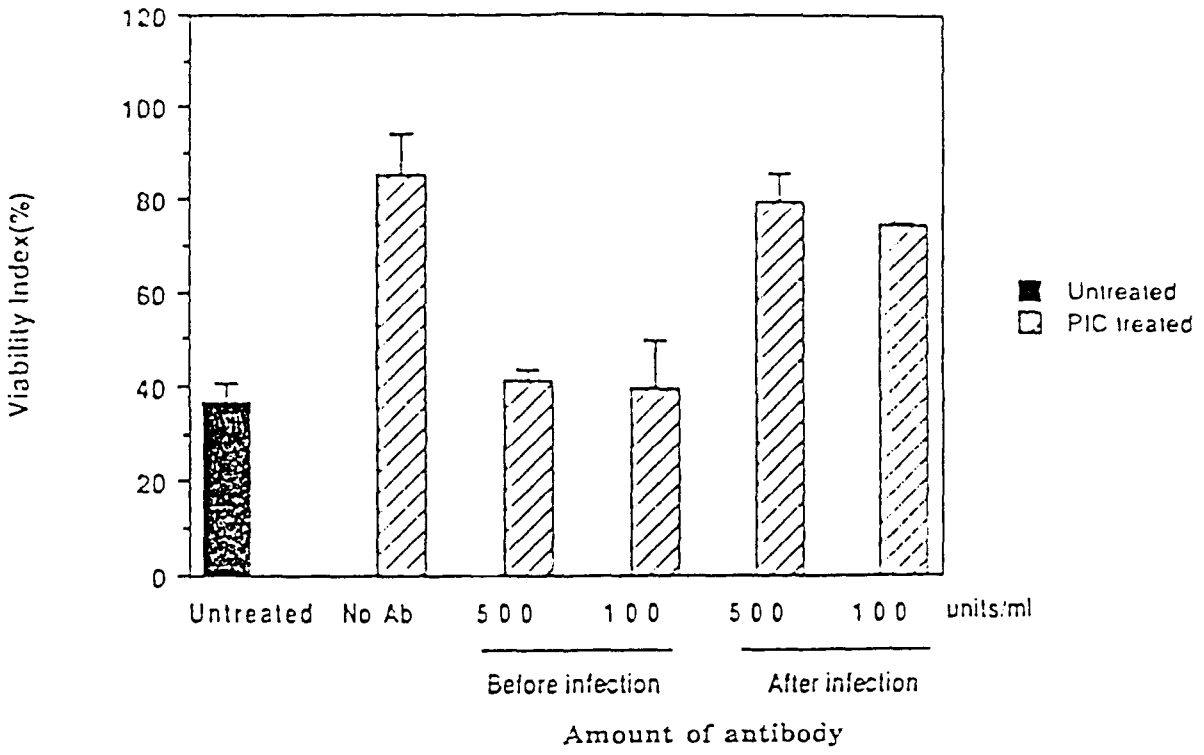


Figure 48. Requirement for IFN-β production prior to virus infection for Poly I:C-induced antiviral activity.

Macrophages were treated with Poly I:C (5 μg/ml) for 24 hours, Poly I:C was removed by washing and the cells were infected with HSV-1 (KOS strain; moi=7). In one group antibodies to IFN-β were added during treatment with Poly I:C (before infection) and in the other group the antibodies were added after infection with virus. CPE was assessed 48 hours later.

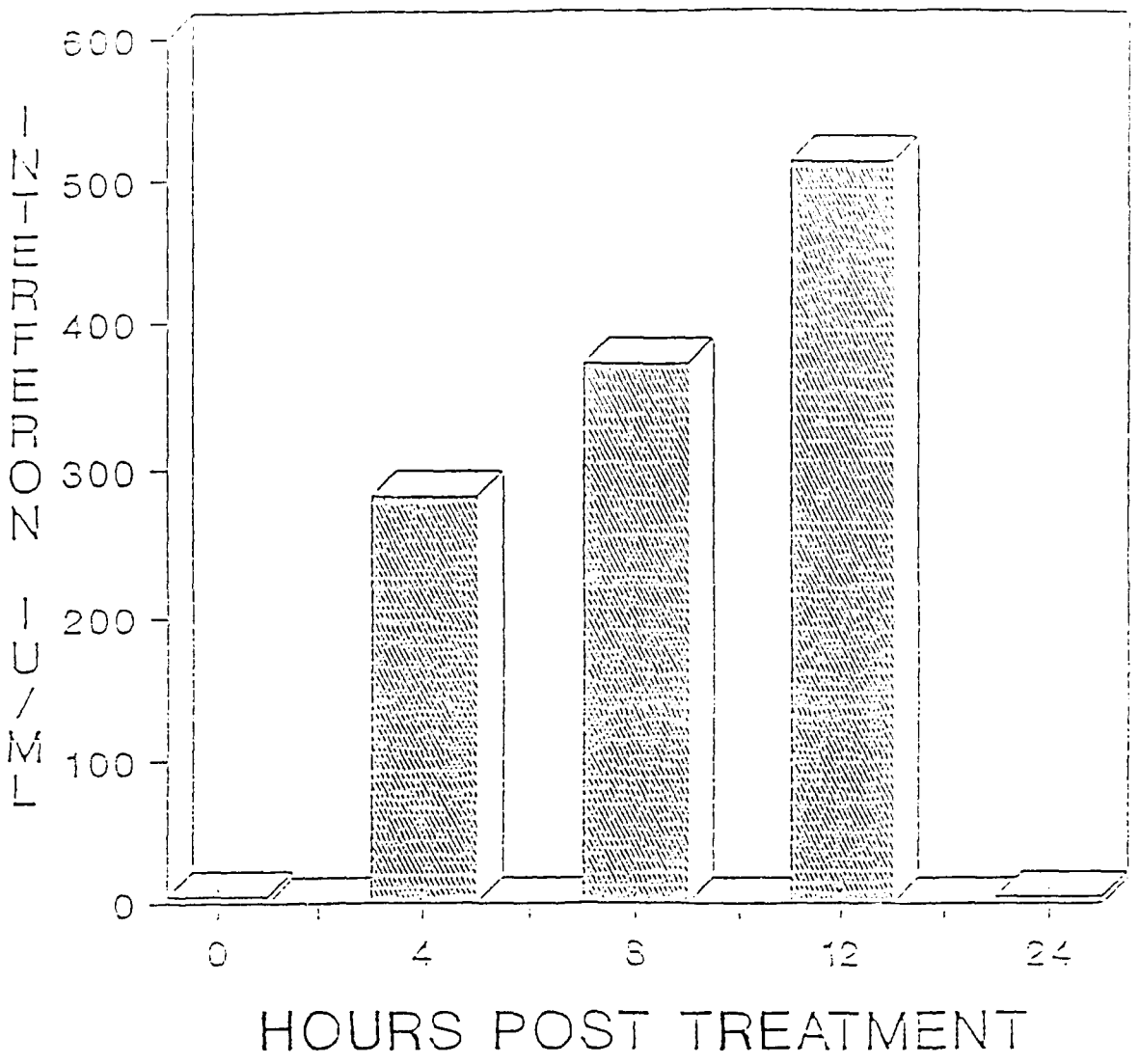


Figure 49. Appearance of serum interferon following poly I:C administration.

Poly I:C (0.5 mg.kg) was administered intravenously. Mice (3 mice/group) were bled at 0, 4, 8, 12, and 24 hours later, and the serum interferon titers determined.

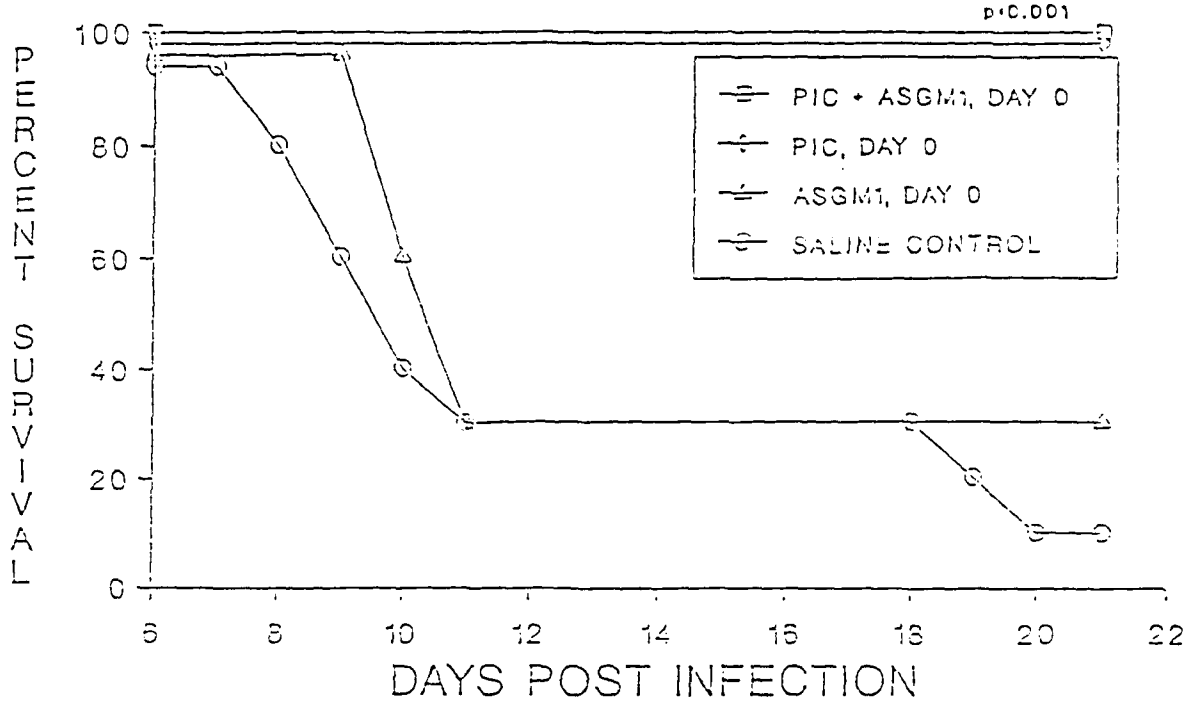


Figure 50. Effect of NK cell depletion on Poly I:C-induced resistance.

C3H/HeN mice, aged 6-7 weeks old, were administered asialo GM1 antibody or saline by intravenous injection 4 hours before treatment with 10 μg Poly I:C and 8 hours before challenge with 2 LD_{100} of Banzi virus.

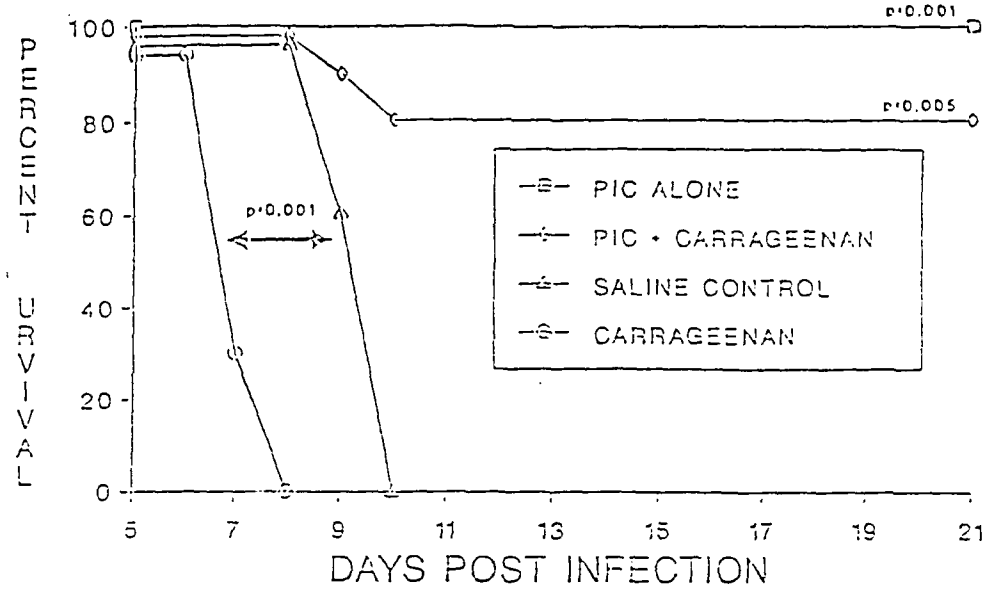


Figure 51. Effect of macrophage blockade on Poly I:C-induced resistance.

C3H/HeN mice, aged 6-7 weeks old, were administered i-carrageenan (0.5 mg) or saline by intravenous injection every 2 days for 6 days, with the final dose 5 days prior to treatment with Poly I:C (10 μg/mouse) and challenge with 2 LD_{100} of Banzi virus.

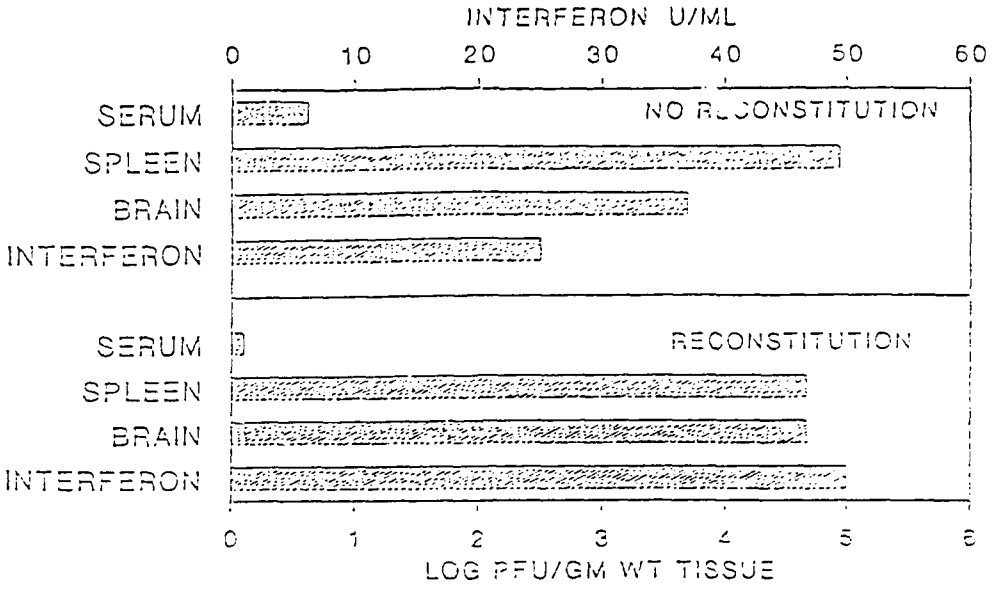


Figure 52. Titers of Banzi virus in tissues of infected mice after adoptive transfer of Poly I:C treated adherent spleen cells.

Groups of four 6-week old mice were given 2.5 x 10^6 Poly I:C treated adherent spleen cells or media i.v. Four hours later mice were challenged with 1 LD_{100} Banzi virus i.p. Six days after challenge, mice were sacrificed and their tissues assayed for virus and serum interferon.

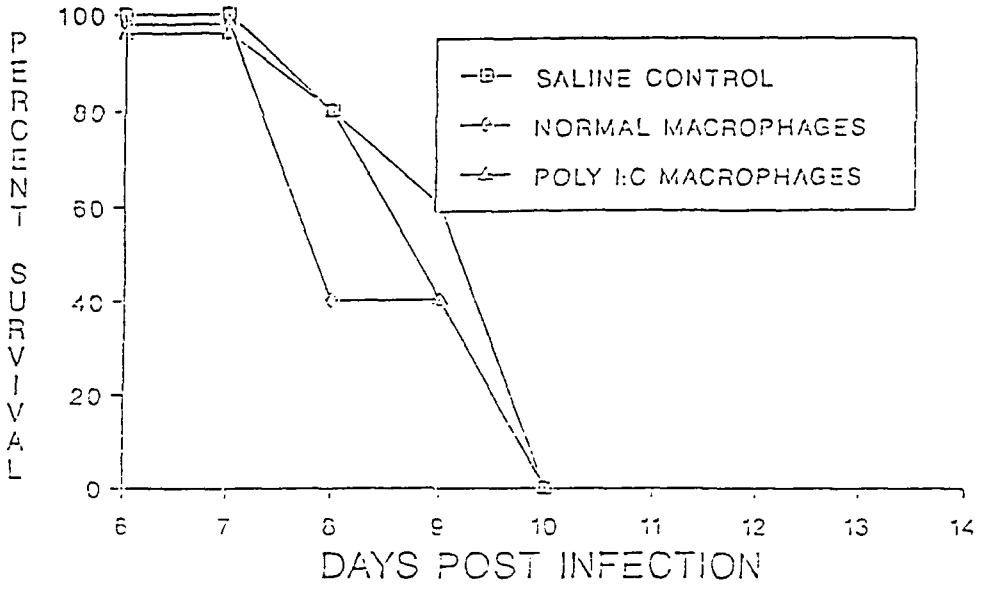


Figure 53. Effect of Poly I:C treated and untreated adherent peritoneal exudate cell transfer on survival.

Groups of ten 6-week old mice were given 1 x 10^7 normal or Poly I:C treated adherent peritoneal exudate cells i.v. Twelve hours later mice were challenged with 1 LD_{100} Banzi virus i.p.

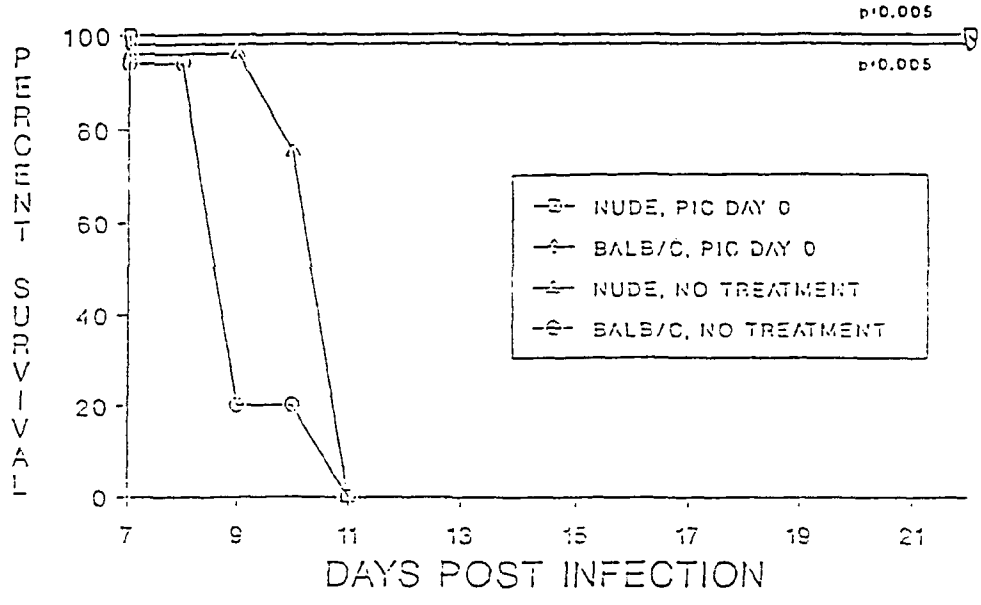


Figure 54. Poly I:C induced resistance in athymic (nu/nu) mice.

Athymic (nu/nu) mice, aged 6-7 weeks old, were administered Poly I:C (10 μg) or saline by intravenous injection 4 hours prior to intraperitoneal challenge with 2 LD_{100} of Banzi virus.

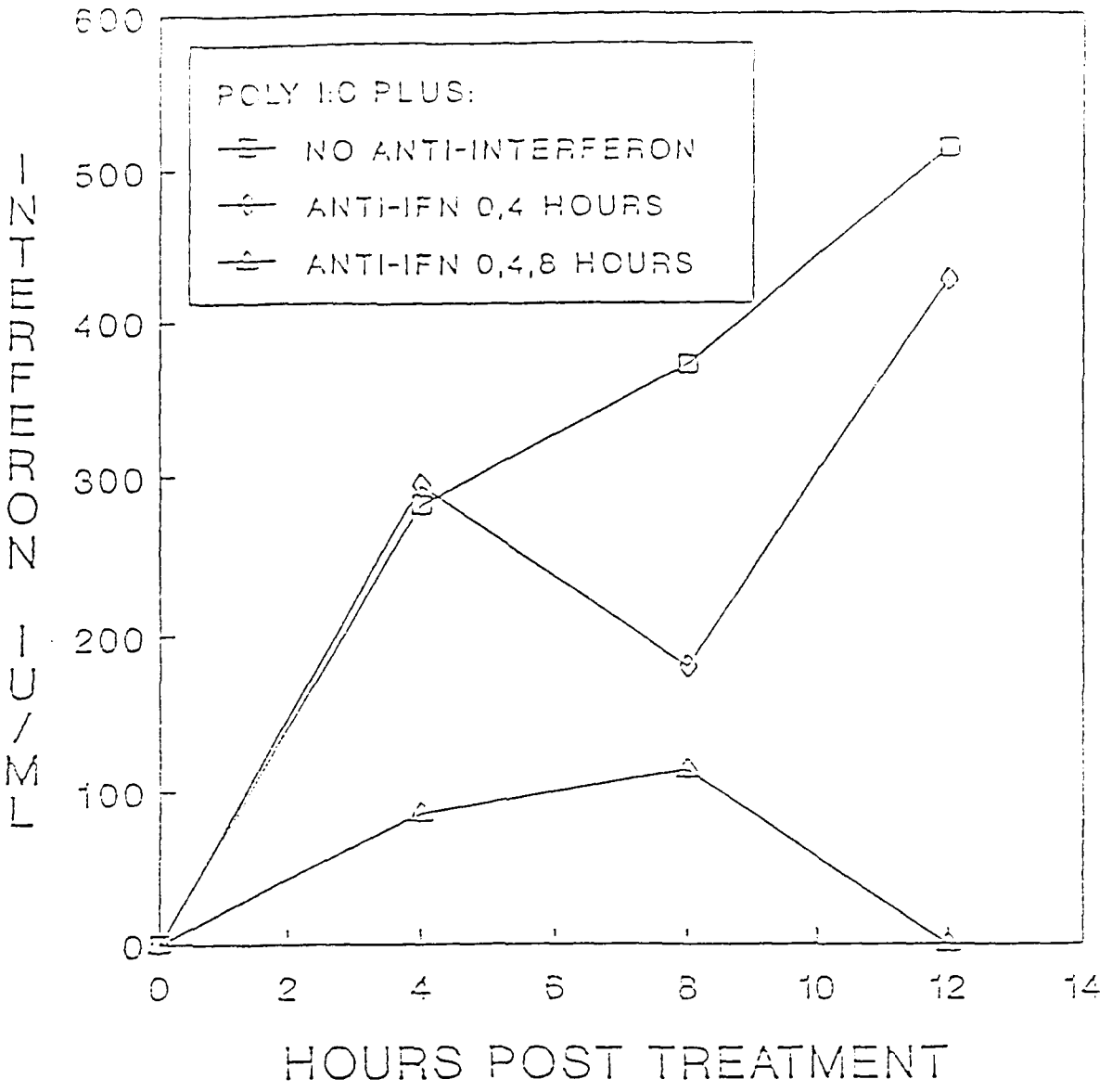


Figure 55. Inhibition of Poly I:C induced serum interferon by anti-interferon antibody.

Animals (3 mice per group) were treated with 10 μg Poly I:C by intravenous injection. Murine antibodies to interferon-α/β were administered i.v. at either 0 and 4 hours or 0, 4, and 8 hours after drug treatment. Mice were sacrificed at the times indicated and serum interferon titers measured.

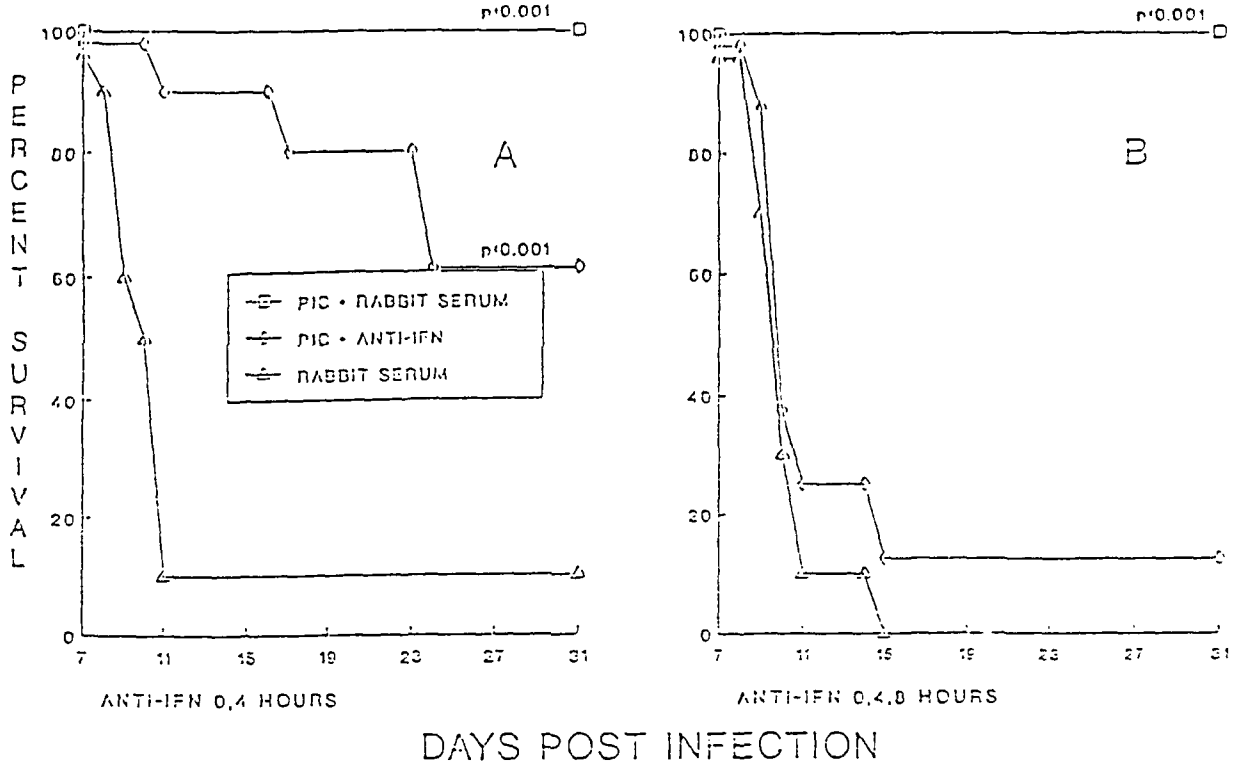


Figure 56. Inhibition of Poly I:C-induced protection by anti-interferon antibody.

C3H/HeN mice (10 mice/group) were treated intravenously with Poly I:C (10 μg/mouse) 4 hours prior to intraperitoneal challenge with Banzi virus at time 0. Antibodies to interferon (1,000 neutralizing units/dose) were given by intravenous injection (1,000 IU/ml per treatment) at 0 and 4 hours (56a), or 0, 4, and 8 hours (56b).

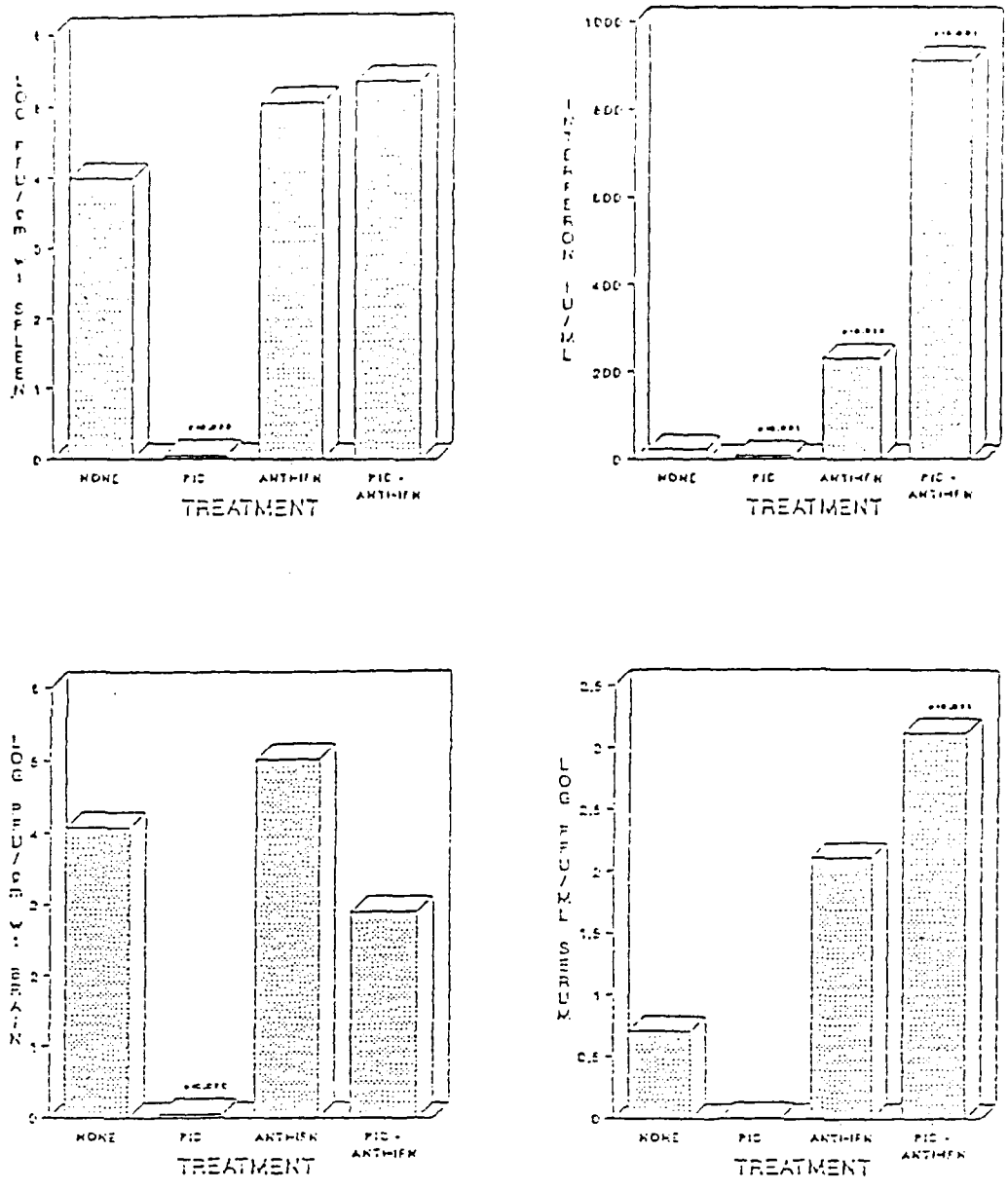


Figure 57. Anti-interferon antibody treatment enhances virus replication.

C3H/HeN mice (10 mice/group) were treated intravenously with Poly I:C (10 μg/mouse) 4 hours prior to intraperitoneal challenge with Banzi virus at time 0. Murine antibodies to interferon-α/β (1,000 neutralizing units/dose) were given by intravenous injection (1,000 IU/ml per treatment) at 0, 4, and 8 hours after infection.

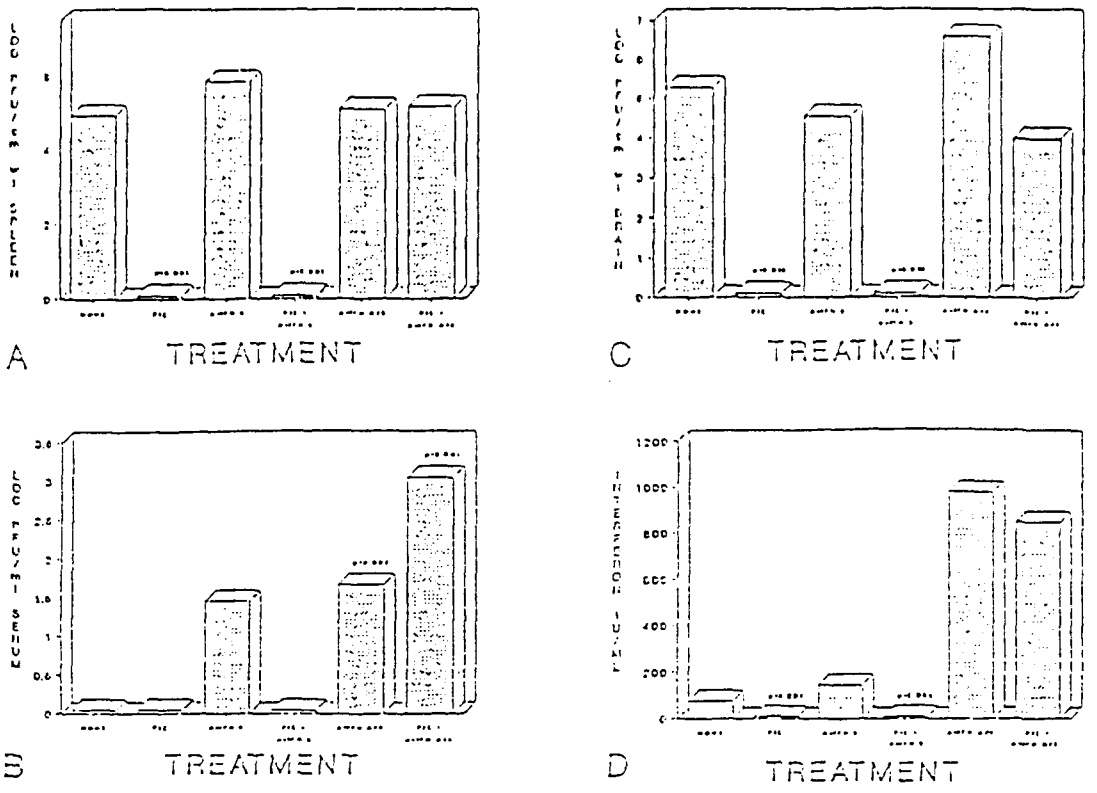


Figure 58. *Neutralization of interferon-β has no effect on virus replication.*

C3H/HeN mice (10 mice/group) were treated intravenously with Poly I:C (10 μg/mouse) 4 hours prior to intraperitoneal challenge with Banzi virus at time 0. Murine antibodies to interferon-α/β or interferon-β (1,000 neutralizing units/dose) were given by intravenous injection (1,000 IU/ml per treatment) at 0, 4, and 8 hours after infection.

PUBLICATIONS

1. Significant Differences in Therapeutic Responses to a Human Interferon-αB/D Hybrid in Rauscher or Friend Murine Leukemia Virus Infections

2. Antiviral Activity of a Novel Recombinant Human Interferon-αB/D Hybrid

3. 9-(2-Phosphonylmethoxyethyl)Adenine in the Treatment of Murine Acquired Immunodeficiency Disease and Opportunistic Herpes Simplex Virus Infections

4. Use of Human Monocytes in the Evaluation of Antiviral Drugs: Quantitation of HSV-1 Cytopathic Effects

5. Drug Targeting: Anti-HSV-1 Activity of Mannosylated Polymer-Bound 9-(2-Phosphonylmethoxyethyl)Adenine

6. Phosphonylmethoxyalkyl Purine and Pyrimidine Derivatives for Treatment of Opportunistic Cytomegalovirus and Herpes Simplex Virus Infections in Murine AIDS

7. Poly I:C-Induced Anti-HSV-1 Activity in Inflammatory Macrophages is Mediated by Induction of Interferon-β

8. Cross-Species Antiviral Activity of a Recombinant Human Alpha-Interferon Hybrid

9. Targeting and Delivery Strategies of Antiviral Substances